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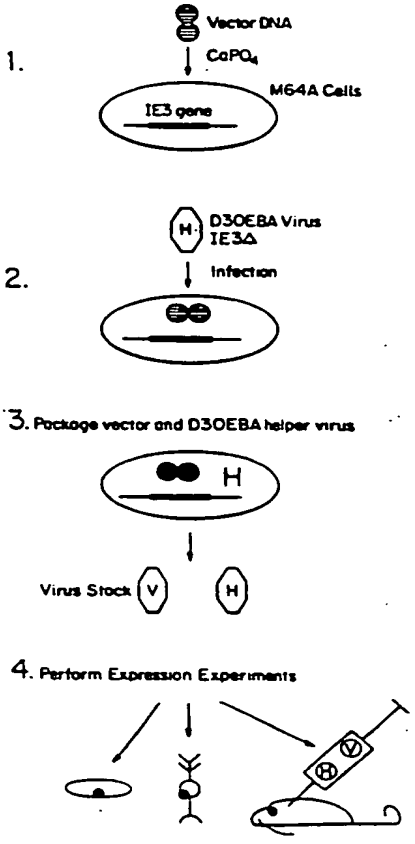
(54) Title: CELL TYPE SPECIFIC ALTERATION OF LEVELS OF GENE PRODUCTS IN NEURAL CELLS

(57) Abstract

A method of altering the level of a gene product in a neuronal or non-neuronal cell, in which a nucleotide sequence encoding the desired gene product is inserted into a defective Herpes virus vector, such as a defective HSV-1 virus vector, in such a manner that gene product expression is under control of the promoter. The resulting defective virus vector construct is packaged into virus particles by introducing the construct into a cell line together with a Herpes mutant helper virus, such as an HSV-1 mutant helper virus, and allowing the virus to propagate. Target cells are infected with the packaged virus construct and the encoded gene product is expressed. In particular, a method of producing tyrosine hydroxylase, nerve growth factor, and several signal transduction factors in neural cells is disclosed.



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CELL TYPE SPECIFIC ALTERATION OF LEVELS OF GENE PRODUCTS IN NEURAL CELLS

Background of the Invention

A defective herpes simplex virus type 1 (HSV-1) vector, pHSV1ac, which contains the E. coli lacZ gene under the control of the HSV-1 Immediate Early 4/5 (IE 4/5) promoter, was recently developed (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988)). The pHSV1ac vector has been propagated and packaged into HSV-1 virus particles, using a temperature sensitive mutant of HSV-1, the HSV-1 strain 17 ts K virus (Davison, M.J. et al., J. Gen. Virol., 65: 859-863, (1984)), following a protocol described by Geller (Geller, A.I. Nucleic Acids Res. 16: 5690 (1988)).

Due to the broad host range of HSV-1, the defective HSV-1 vector can be introduced into a wide variety of cells. HSV-1 can also infect postmitotic cells, including neurons in adult animals, and can be maintained indefinitely in a latent state (Stevens, J.G. et al., Curr. Top. Microbiol. Immunol., 70: 31-50 (1975)). In particular, infection with the temperature sensitive virus results in

persistence of the virus without productive infection at the non-permissive temperature, and prevents cell death (Watson et al., J. Gen. Virol. 49: 149-159 (1980)). The IE 4/5 promoter present in pHSV1ac functions in most cell types, and upon infection of various neural cell lines (Geller et al., Abstr. Soc. Neurosci. 14: no. 254.11 (1988)) and a variety of human cell types (Boothman, D. A. et al., FEBS Lett. 258: 159-162 (1989)), the pHSV1ac virus has been shown to direct expression of the LacZ gene product, β-galactosidase.

Summary of the Invention

The present invention relates to a method of altering the level of a gene product in a neuronal or non-neuronal cell, comprising inserting a nucleotide sequence encoding the desired product into a defective Herpes virus vector, such as a defective HSV-1 virus vector, such that the promoter of the vector is able to express a functional gene product upon introduction of the virus vector into the cell. The resulting defective virus vector construct, encoding the gene product of interest, is packaged into virus particles by introducing the construct into a cell line together with a neurotropic Herpes mutant helper virus, such as an HSV-1 mutant helper virus, and allowing propagation of the virus. Target cells (i.e., cells in which expression of the gene product is desired) are infected with the packaged virus (i.e., the packaged virus construct), and a functional gene product is

expressed. A functional gene product, which can be an RNA, a protein or a peptide, is one which has an activity of the desired product.

In particular, the invention relates to a method of packaging an HSV-1 defective virus vector into virus particles using a deletion mutant virus as helper virus. The HSV-1 defective virus vector and an HSV-1 deletion mutant helper virus are introduced into a complementing cell line. The cell line contains Herpes virus sequences and is able to complement the defect of the deletion mutant virus so that the HSV-1 defective virus vector is packaged into virus particles.

The invention further relates to a method of altering the level of a gene product in cells to alter a disease state. In particular, a defective HSV-1 virus vector, pHSVth, capable of expressing human tyrosine hydroxylase is described, and has been shown to increase the level of functional tyrosine hydroxylase in a cell. Production of tyrosine hydroxylase in this manner is useful for altering the level of this enzyme in cells affected by Parkinson's disease.

In the method of the present invention, the level of a gene product can be altered in a cell by introducing into the cell a vector in which a sequence encoding the gene product is under the control of a cell type-specific promoter. Expression of the gene product is directed by the promoter in a cell-type-specific manner. For example, the human neuronal cell-specific neurofilament L promoter can be used in a defective Herpes virus vector for this purpose.

Furthermore, the gene product can be targeted to a particular location in the cell (e.g. a neuronal process) by use of an intracellular targeting sequence, such as the amino terminal region of human GAP-43, a growth-associated protein, which is localized to nerve tissue and linked to the synaptosomal membrane. The intracellular targeting sequence is incorporated into the nucleotide sequence encoding the gene product to be expressed from the defective Herpes virus vector in a location consistent with its targeting function.

The present invention further relates to a method of altering neurotransmitter metabolism in a cell by altering the level of a gene product, which alters neurotransmitter metabolism, in the manner described. In particular, neurotransmitter metabolism (e.g., neurotransmitter release) is altered in non-neuronal (e.g., glia, fibroblasts) and neuronal (e.g., striatal neurons, sympathetic neurons) cells by altering the level of a gene product, such as human tyrosine hydroxylase, nerve growth factor or a gene product that participates in a signal transduction or second messenger pathway, or fragment thereof, such as adenylate cyclase. The invention also pertains to the particular defective HSV-1 virus vectors useful in the present method.

Brief Description of the Drawings

Figure 1 illustrates the structure of the pHSVlac vector, which contains the HSV-1 IE 4/5 promoter (arrow), the intervening sequence following

that promoter (triangle), the LacZ gene, SV40 early region polyadenylation site, the HSV-1 ori_s (small circle) from the HSV-1 c region, the HSV-1 a sequence containing the packaging site, and sequences from pBR322 which allow propagation and selection of pHSV1lac DNA in E. coli. The three Eco RI sites (RI) of the vector are indicated.

Figure 2 illustrates the structure of HSV-1 DNA in M64A cells and D30EBA deletion helper virus. The top line is a schematic map of the HSV-1 genome. The IE-3 gene is present in two copies in the duplicated c region. The second line diagrams the HSV-1 DNA present in the genome of M64A cells. The fragment is flanked by Xho I (X) and Sma I (S) sites, and contains the IE-3 gene encoding 1298 amino acids. The third line indicates the extent of the deletion (codons 83-1236) in the IE-3 gene in D30EBA virus. The fourth line represents the 659 bp fragment used in Southern analysis of the viral DNA.

Figure 3 illustrates (1) calcium phosphate DNA transfection of defective HSV-1 vector DNA into M64A cells (complementing cell line), which contain the IE-3 gene; (2) superinfection of transfected cells with D30EBA helper (H) virus; (3) the IE-3 gene in M64A cells complements the D30EBA virus, resulting in productive infection and release of virus particles containing packaged D30EBA (H) or HSV-1 vector virus (V); (4) the virus stock is used to infect cells in culture, such as neurons or glia, or to infect cells by stereotactic injection into the brain of a rat, for example.

Figure 4 illustrates the structure of defective HSV-1 vector pNFLlac. The location of the human neurofilament L (hNFL) promoter is indicated.

Figure 5 illustrates the structure of defective HSV-1 vector pHSVGAPlac. G10 indicates the nucleotide sequence of the GAP-43 intracellular targeting sequence.

Figure 6 illustrates the structure of defective HSV-1 vector pHSVth, which carries the human tyrosine hydroxylase (TH) gene.

Figure 7 illustrates the structure of defective HSV-1 vector pHSVcyr, and a schematic map of the yeast adenylate cyclase protein from the amino (N) to the carboxyl terminus (C), indicating the locations of the regulatory and catalytic portions of the protein. The nucleotide sequence encoding the catalytic portion is present in pHSVcyr.

Figure 8 illustrates the structure of the 5'-end of the gpt-trpS-lacZ fusion of pHSVlac. Restriction sites for Hind III and Asp718 are indicated.

Figure 9 is a diagram illustrating the structure of the pHSVngf vector. The vector contains 2 genetic elements, the HSV ori$_s$ and pac a sites (HSV pac), that are necessary for packaging into viral particles. It also contains a transcription unit composed of the HSV IE 4/5 promoter and the NGF mini-gene with SV40 polyadenylation signal (SV-40 poly A). For propagation in E. coli the vector contains a Col E1 origin of replication and the β-lactamase gene (Col E1 ori, Amp).

Figure 10 is a histogram illustrating the effect of injection of pHSVngf virus particles on tyrosine hydroxylase (TH) activity (pmole product/ganglion hour) in the axotomized rat superior cervical ganglion (SCG) as compared with injection of pNFlac virus particles. In each rat, the right SCG was neither injected or axotomized and served as an internal control. Ten rats were injected with pHSVngf virus and nine rats were injected with pNFlac virus particles. The data are shown as a mean +/- S.E.M. TH activity for each group of ganglia. From left to right the groups of ganglia were treated by: pHSVngf injection + axotomy; control, (right SCG control from animals receiving pHSVngf virus); pNFlac injection + axotomy; and control, (right SCG control from animals receiving pNFlac virus).

Figure 11 is a histogram illustrating the effect of pHSVngf virus infection on the choline acetyl transferase (ChAT) activity of striatal cholinergic neurons. From left to right the bars correspond to: (a) mock infected control; (b) uninfected cells with 100 ng/ml exogenous NGF added; (c) pHSVlac virus infected cells; (d) pHSVngf virus infected striatal cells (5 μl virus stock); and (e) pHSVngf virus infected striatal cells (10 μl virus stock).

Detailed Description of the Invention

The present invention relates to a method of altering the level of a gene product in a cell, comprising inserting a nucleotide sequence encoding

the desired product into a defective Herpes virus vector, such that the vector is able to express a functional gene product upon introduction of the vector into the cell. A defective Herpes simplex type 1 (HSV-1) vector, pHSV1ac, which contains the *E. coli Lac Z* gene under the control of the HSV-1 Immediate Early 4/5 (IE 4/5) promoter, was recently developed. The construction and structure of the defective HSV-1 vector, pHSV1ac, is described in detail in U. S. Serial No. 304,619, filed February 1, 1989, and in papers by Geller and Breakefield (Geller, A.I. and Breakefield, X.O., *Science* 241: 1667-1669, (1988)) and by Geller and Freese (Geller, A.I. and Freese, A., *Proc. Natl. Acad. Sci. USA*, 87: 1149-1153 (1990)), the teachings of each of which are incorporated herein by reference.

The HSV-1 vector pHSV1ac (Figure 1) contains the *E. coli Lac Z* gene under the control of the HSV-1 Immediate Early 4/5 (IE4/5) promoter. (The intervening sequence following the IE4/5 promoter is also present.) In addition, the SV40 early region polyadenylation site is incorporated 3' to the *lacZ* gene. The backbone of the vector contains the *Col* E1 origin of replication and the gene conferring ampicillin resistance for propagation and selection in *E. coli*. An HSV-1 origin of replication, ori_s and the HSV *a* sequence, which contains the virus packaging site, are also present. The latter two portions of pHSV1ac allow propagation and packaging of pHSV1ac in the presence of HSV-1 helper virus.

In previous studies, the pHSV1ac vector was propagated and packaged into HSV-1 virus particles using a temperature sensitive (ts) mutant of HSV-1, the HSV-1 strain 17 ts K virus (Davison, J.J. et al., J. Gen. Virol., 65:859-863 (1984)), in a protocol described by Geller (Geller, A.I., Nucleic Acids Res. 16: 5690 (1988)). The ts K mutant virus has a single base change in a gene essential for the HSV-1 lytic cycle, the IE-3 gene. The mutation results in an amino acid substitution in the encoded protein, which renders the virus incapable of undergoing the lytic cycle at 37-39°C. The mutant virus is able to propagate at the permissive temperature of 31°C. Thus, the virus can be used as a helper virus to propagate and package defective HSV-1 vectors at the permissive temperature. On infection of a cell at the non-permissive temperature of 37°C, with the virus stock containing both helper virus particles and particles containing the defective HSV-1 vector, the ts helper virus is unable to sustain a productive lytic infection, and persists without productive infection. Thus, the vector is delivered to the cell and persists without a productive cytopathic infection. However, in practice, the HSV-1 strain 17 ts K mutants revert to wild type, and the temperature sensitive phenotype is not absolute at 37 °C, making these viruses unsuitable as helpers for packaging defective HSV-1 vector constructs for gene therapy in human hosts, for example.

In contrast, in an embodiment of the present invention, a deletion mutant virus packaging (i.e., propagation and packaging) system is used. Herpes deletion mutant helper viruses suitable in the present invention will contain a deletion of all or part of a gene essential for productive lytic infection by the virus. A deletion may be the absence of two or more nucleotides that results in an unconditional mutation, such that productive lytic infection of the deletion virus in a cell can result only when all or part of the deleted sequences or some other complementing sequences or factor(s) are provided in the cell so that the defect is complemented. An HSV-1 deletion mutant helper virus with a deletion of all or part of the IE-3 gene product, the major regulatory gene of HSV-1, is preferred. After packaging, introduction of the deletion mutant helper virus into a target or other cell, alone or together with the desired defective Herpes virus vector, will not damage the cell because the deletion mutant viruses essentially do not revert.

To use a deletion mutant virus to package a defective Herpes virus vector, a complementing cell line is constructed, which complements the defect of the deletion mutant helper virus, allowing production of viral particles. A defective Herpes virus vector construct, encoding a gene product of interest, can be introduced into a complementing cell line by transfection (Graham, F.L. and Van der Eb, A.J., Virology 52: 456-467 (1973)) or other

suitable methods. The Herpes deletion mutant helper virus can be introduced into the cells by superinfection of the transfected complementing cell line, for example (Geller, AI., Nucleic Acids Res. 16: 5690 (1988)). Deletion viruses may be constructed as described by DeLuca et al. and Paterson and Everett (DeLuca et al. J. Virol. 56: 558-570 (1985); Paterson, T. and Everett, R.D., J. Gen. Virol. 71: 1775-1783 (1990)). Complementing cell lines may be derived from a cell line capable of supporting infection of the helper virus. For example, a BHK TK$^-$ fibroblast line is suitable for use with HSV-1, as described (Davidson, I. and Stow, N.D., Virology 141: 77-88 (1985); Paterson, T. and Everett, R.D., J. Gen. Virol. 71: 1775-1783 (1990)).

For example, the D30EBA HSV-1 deletion virus and complementing cell line M64A described by Paterson and Everett (Paterson, T. and Everett, R.D., J. Gen. Virol 71: 1775-1783 (1990)) are useful in the present method (Figure 2, Figure 3). The D30EBA helper virus has a deletion in the same gene that is mutated in the HSV-1 ts K helper virus. The deletion in D30EBA encompasses codons 83-1236 of the 1298 codon IE-3 gene. Defective HSV-1 vectors pHSVlac and pHSVth (which carries the gene for human tyrosine hydroxylase), pNFLlac (in which the human neurofilament L promoter is operably linked to Lac Z), and pHSVGAPlac (in which an intracellular targeting sequence is fused to Lac Z), were efficiently packaged with D30EBA helper virus in the M64A cell line, and the packaged virus was used to

infect and then express gene products in various cell types.

Notably, the D30EBA helper virus reverts at a much lower frequency than the ts K helper virus. The reversion frequency of D30EBA, grown in the presence of pHSV1ac DNA, was about 5 X 10^{-5} compared to the apparent reversion rate of about 2 X 10^{-3} for the ts K helper virus grown at the non-permissive temperature of 37°C (including incomplete penetrance of the ts K mutation at this temperature). In addition, although D30EBA and the ts K helper both have mutations in the IE-3 gene and have the same immediate early phenotype, pHSV1ac virus (i.e., pHSV1ac packaged into HSV-1 particles) titers were 25-fold greater using the deletion mutant as helper than when the ts K helper virus was used.

The reversion frequency of the deletion virus is probably due to homologous recombination between the deletion virus and the HSV-1 DNA flanking the IE-3 gene in the M64A complementing cell line. This recombination could be reduced or eliminated by construction of a helper line with less extensive or no homology to the deletion mutant in the region flanking the deletion or by increasing the extent of the deletion in the helper virus, or both. Furthermore, deletion mutants with one or more additional mutations, particularly deletions, in genes required for productive HSV-1 infection could be used to further reduce reversion. In these strains, the complementing cell line would be capable of complementing each virus defect.

Other herpes mutant viruses can be used as helper virus, such as neurotropic Herpes mutant helper viruses. A neurotropic Herpes virus is one that is capable of infecting neural cells; although such a neurotropic Herpes virus (e.g. HSV-1, Herpes simplex virus type-2 (HSV-2), and pseudorabies virus) may also be able to infect non-neural cells. The Herpes mutant helper virus is a mutant virus, incapable of productive lytic infection in the target cells. Preferably, the Herpes mutant helper virus does not revert (i.e., becomes altered in a manner that confers the ability to direct a productive lytic infection in the target cell) when maintained under the conditions permissive for productive infection, such as in an appropriate cell line, during packaging of the Herpes virus vector into virus particles. The defective Herpes virus vector can be derived from any Herpes virus, or combination of Herpes viruses, providing the vector can be encapsidated into a Herpes virus particle by a Herpes helper virus.

In one embodiment of the present invention, the nucleotide sequence of a desired gene product(s) is introduced into a defective HSV-1 vector backbone. The gene product(s) can be RNA transcribed from the nucleotide sequence (e.g., an anti-sense RNA), protein(s) and/or peptide(s) encoded by the nucleotide sequence, or portions thereof. The nucleotide sequence can be, for example, a foreign sequence, synthetic DNA, genomic DNA or cDNA sequence that is operably linked to a promoter in

the defective HSV-1 vector, such that the desired functional gene product(s) is expressed, resulting in alteration (generally an increase) in the level of the encoded gene product.

The IE 4/5 promoter present in pHSV1ac functions in most cell types. Upon infection of various neural cell lines (Geller et al., Abstr. Soc. Neurosci. 14, no. 254.11 (1988)) and a variety of human cell types, including tumor cell types (Boothman, D. A. et al., FEBS Lett. 258: 159-162 (1989)), the pHSV1ac virus has been shown to direct expression of the Lac Z gene product, β-galactosidase. In one embodiment of the present invention, the level of a gene product can be altered in a cell type-specific manner, by introducing into the cell a defective Herpes virus vector in which a sequence encoding the gene product is under the control of a cell type-specific promoter. Expression of the gene product is directed by the promoter in a cell type-specific manner. Thus, although a broad range of cell types may be infected, the desired gene product, when under the control of a cell type-specific promoter, can be expressed preferentially or exclusively in one or more specific cell types. The range of cell types (cell type specificity) will vary depending on the nature of the promoter or promoter fragment used. However, in each case, the range of expression of the gene product will be restricted (i.e., promoter is active in fewer cell types) and/or will display an altered specificity (i.e.,

the level of activity is altered, either increased or reduced, in certain cell types) as compared to the IE 4/5 promoter. Thus, expression of the gene product in cells may be directed to the appropriate cell types.

In one embodiment, a fragment containing the neuronal cell-specific human neurofilament L promoter (Julien, J.P. et al., Genes and Dev. 1: 1085 (1987)) is introduced into a defective HSV-1 vector backbone to make pNFLlac (Figure 4). This defective HSV-1 vector construct was packaged by a deletion mutant helper virus, and directed cell type-specific expression of a functional gene product encoded by the nucleotide sequence under its control in the vector, upon introduction into cells pNFLlac construct by infection. Expression is preferentially activated in neurons by the neurofilament L promoter in the construction.

Other cell type-specific promoters are useful in the present invention, such as the voltage-gated Na^+ channel II promoter or the neuronal specific enolase promoter, which are both cell type-specific promoters active in neuronal cells. The vasoactive intestinal peptide (VIP) promoter (Linder, S. et al., Proc. Natl. Acad. Sci. USA 84:605-609 (1987)) can be used to restrict expression of the gene product to neurons which use the VIP neurotransmitter, for example. In another embodiment, a cell type-specific promoter that is active in dividing cells but not quiescent cells, such as the promoter from an appropriate cell cycle regulated gene, can

be used to drive cell type-specific expression of a cytotoxin (e.g., ricin) from a defective HSV-1 vector in neural tumor cells to kill the cells.

Furthermore, the gene product can be targeted to a particular location in the cell (e.g., the cell body, nucleus, or neuronal processes) if desired, by use of an intracellular targeting sequence, such as the human GAP-43 targeting sequence. The nucleotide sequence encoding the intracellular targeting sequence is incorporated into the nucleotide sequence of the gene product to be expressed from the defective HSV-1 virus vector in a location consistent with its targeting function.

GAP-43 is a neuronal growth-associated protein and is linked to the synaptosomal membrane. It is also a major protein of the growth cone membrane complex. GAP-43 is thought to be attached to the growth cone membrane via fatty acylation of the protein's only two cysteine residues. These residues are found within the N-terminal 10 amino acids of GAP-43, and the N-terminal 10-40 residues are thought to be involved in targeting the rat GAP-43 protein to cellular processes (Kosik, K.S. et al., Neuron 1: 127-132 (1988); Zuber et al., Nature 341: 345-348 (1989)).

For example, a GAP-43 targeting sequence, which is a nucleotide sequence of a portion of the human GAP-43 coding sequence, or a functional equivalent thereof, capable of targeting a gene product to neuronal processes, could be incorporated into the nucleotide sequence of the desired gene product to

direct the gene product to neuronal processes. Nucleotide sequences encoding variants of such a targeting sequence that retain targeting function are included in the present method, and such variants are considered to be GAP-43 targeting sequences. The N-terminal 10 codons of human GAP-43 can be incorporated into the nucleotide sequence of the desired gene product, as in pHSVGAPlac (Figure 5) to target a gene product to neuronal processes. In this construct, a nucleotide sequence specifiying the N-terminal 10 codons of human GAP-43 is fused in frame to nucleotide sequence specifying the N-terminus of the gene product. It is possible that incorporation of the nucleotide sequence specifying a GAP-43 targeting sequence in another location of the nucleotide sequence of the desired gene product will also result in targeting to neuronal processes. Furthermore, additional sequences from GAP-43, either contiguous or non-contiguous with the N-terminal 10 codons in GAP-43 may enhance the efficiency of targeting or confer specificity of targeting to axonal processes. These sequences may be incorporated into the nucleotide sequence of the desired gene product in one or more locations consistent with the targeting function. Alternatively, a targeting sequence derived from another molecule that is localized to a particular portion of a cell and that is capable of targeting the gene product to the desired intracellular location when incorporated into the nucleotide

sequence of the gene product in one of the manners described can be used.

The invention further relates to a method of altering the level of a gene product in target cells to alter a disease state or an undesired or abnormal condition. Expression of a desired gene product in a target cell can act, directly or indirectly, to prevent, reduce, or reverse a disease process. For example, the gene product can act on the target cell to correct a defect in that cell associated with a disease process or can alter a disease state in another cell. In the latter case, for example, altering the level of the gene product in the target cell can result in secretion of a substance or substances (e.g., neurotransmitters, growth factors) that act, directly or indirectly, to alter a disease state that affects the other cell or can induce cell-cell interactions that alter the disease state. For example, defective HSV-1 vectors can be used to introduce a gene product into a wide variety of cell types, including postmitotic cells such as neural cells (e.g., neurons, glia) to affect neurological disorders such as Parkinson's disease or Alzheimer's disease.

Parkinson's disease is a neurodegenerative disorder resulting from the destruction of dopaminergic neurons in the substantia nigra pars compacta, which project into the corpus striatum (Yahr, M.D. and Bergmann, J (Eds.), Parkinson's Disease, Raven Press, New York, (1987)). As tyrosine hydroxylase is the rate-limiting enzyme in dopamine

biosynthesis, introduction of the tyrosine hydroxylase gene into neurons in, or projecting to, the striatum increases striatal dopamine levels.

In the method of the present invention, tyrosine hydroxylase (TH) can be introduced into neural (e.g., neurons, glia) or neuronal cells to alter a disease state, such as Parkinson's disease. A nucleotide sequence encoding TH can be introduced into a defective Herpes virus vector such that it is under the control of a promoter (e.g., a neuronal cell-specific promoter) in the vector, and functional TH can be expressed. The resulting defective Herpes virus vector is introduced into the desired cells by infection, following packaging, in the method of the present invention, and the level of the TH gene product in the cells is altered. A nucleotide sequence encoding tyrosine hydroxylase, a functional equivalent, or portion thereof, could be used to produce TH. Nucleotide sequences encoding variants or portions of TH that retain TH function are included in the present method, and such variants or portions of TH are included in the designation "tyrosine hydroxylase".

In one preferred embodiment, a cDNA fragment encoding human tyrosine hydroxylase (O'Malley, K.L. et al., Biochemistry 26: 6910-6914 (1987) is introduced into a defective HSV-1 vector backbone, such that it is under the control of a promoter in the vector, to make pHSVth (Figure 6). Using pHSVth in the method of the present invention, pHSVth was able to direct expression of active TH in cells in

culture, and in neuronal cells (neurons), such as striatal neurons, pHSVth was able to induce an increase in monoamine neurotransmitter release (e.g., L-DOPA and dopamine).

In a further embodiment of the present invention, the level of a gene product in cells which do not normally (naturally) express the product can be altered. For example, a neurotrophic factor such as nerve growth factor can be introduced into cells (e.g., neural cells) to alter (e.g., reduce or prevent) a disease state (e.g., diabetes, Alzheimer's disease) or to prevent, reduce or reverse the effects of injury to the nervous system (e.g., traumatic axon injury, neurotoxicity). A defective Herpes virus construct which encodes a neurotrophic factor (e.g., nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), ciliary neurotrophic factor (CNTF), basic or acidic fibroblast growth factor (FGF)) can be used to introduce a neurotrophic factor into a cell which normally expresses the factor or which does not normally express the factor. Because neurotrophic factors are secreted proteins, their action is not limited to the cells that synthesize the factor. Thus, the expression of a neurotrophic factor gene from an HSV vector should result in an effect on a greater number of responding cells (i.e., cells having an appropriate receptor) than the number of infected and expressing cells. If the cell expressing a neurotrophic factor also expresses

an appropriate receptor, it too can be affected by the factor by an autocrine mechanism.

Each member of the family of neurotrophic factors, including factors such as nerve growth factor, brain derived neurotrophic factor, and neurotrophin 3, promotes the survival of particular types of developing neurons (NGF, Thoenen, H. et al., Rev. Physiol. Biochem. Pharmacol. 109: 146-178 (1987); BDNF, (Lindsay, R.M. et al., Dev. Biol. 112: 319-328 (1985); Hofer, M.M. and Y. Barde, Nature 331: 261-262 (1988); NT-3, (Hohn, A. et al., Nature 344: 339-341 (1990); Rosenthal, A. et al., Neuron 4: 767-773 (1990)). Defective HSV-1 vectors that express other neurotrophic factors, such as BDNF, neurotrophin-3, neurotrophin-4, basic or acidic fibroblast growth factor or CNTF (Stockli, K. et al., Nature 342: 920-922 (1989)) are useful in the present invention for maintaining neuronal phenotype and promoting neuronal survival to prevent, reduce or reverse disease or the effects of injury. In addition, the ability to use such viruses to alter the levels of specific neurotrophic factors in specific regions of the brain (e.g., the basal forebrain) will contribute to the understanding of the functions of these factors in the normal, diseased or injured nervous system, and will facilitate the development of the therapeutic potential of neurotrophic factors in the diseased or injured nervous system.

In one particular embodiment, a nerve growth factor (NGF) minigene was constructed and inserted into a defective HSV-1 vector to make pHSVngf. This

construct was packaged and used to infect cells which do not normally or naturally express NGF (e.g., fibroblasts, sympathetic neurons). The cells produced biologically active NGF, which was able to prolong the survival of sympathetic neurons.

The superior cervical ganglion (SCG) of adult rats contains neuronal and non-neuronal cells (sympathetic neurons and surrounding glia) which do not express NGF. The sympathetic neurons in the SCG of adult rats depend on target-derived nerve growth factor for maintenance of tyrosine hydroxylase levels and the noradrenergic neurotransmitter system. Axotomy of a SCG results in NGF deprivation, causing a decline in TH activity in the sympathetic neurons; however, continuous local application of NGF can prevent this decline in TH activity. Injection of a defective HSV-1 vector that expresses NGF (pHSVngf) into superior cervical ganglia in vivo prevented the decline in TH activity that follows axotomy. Moreover, in one experiment, an 18% increase in TH levels relative to control ganglia was observed in ganglia injected with pHSVngf. Therefore, HSV-1 vectors can be used to prevent deleterious effects (e.g., decline in TH levels) of nerve injury (axon injury) in vivo. The ability to increase the neurotrophic factor supply to neurons, such as neurons deprived of neurotrophic factors, by the use of defective HSV-1 vectors capable of expressing genes encoding neurotrophic factors provides a method of preventing, reducing or reversing the effects (i.e., treating) of peripheral neural injury.

Disruption of neurotrophic factor supply in the central nervous system (CNS) may also produce disease. For example, in the adult CNS, a trophic role for NGF has been demonstrated for the group of ascending basal forebrain cholinergic neurons that synapse on NGF-producing hippocampal neurons. When these cholinergic neurons are disconnected from their NGF-producing target cells by axotomy, they degenerate and their content of choline acetyltransferase (ChAT) decreases. Disruption of this NGF supply may be involved in pathophysiology of Alzheimer's Disease, which is characterized by a progressive loss of cognitive function which is correlated with degeneration of cholinergic neurons (affected cells) in the basal forebrain (reviewed in Hefti, F. et al., Neurobiol. Aging 10: 515-533 (1989)).

In addition, disruption of NGF supply from targets of innervation to neuronal cell soma is observed after administration of pharmacological agents that interrupt axonal transport, and in metabolic disorders such as diabetes. NGF deprivation may be a direct or indirect cause of toxic or metabolic neuropathy, as well as neuropathy secondary to injury. Maintenance of the NGF supplies of the neurons affected in these conditions by the method of the present invention can prevent, reduce or reverse damage to the neurons associated with these conditions.

In a method of the present invention, nerve growth factor can be introduced into neural cells to alter a disease state such as Alzheimer's disease.

A nucleotide sequence encoding nerve growth factor can be introduced into a defective Herpes virus vector under the control of a promoter in the vector, and functional NGF can be expressed from the resulting construct. Following packaging by the method of the present invention, the packaged virus construct is used to infect target cells, and the level of NGF in those cells is altered. NGF can affect the target cell (e.g., an affected cell, or a cell which can secrete NGF to alter a disease state affecting another cell) to reduce the symptoms of the disease. A nucleotide sequence encoding nerve growth factor, a functional equivalent, or functional portion thereof, could be used to produce NGF. Nucleotide sequences encoding variants or functional portions of NGF are useful in the present method and are included in the designation "nerve growth factor". For example, the nerve growth factor "minigene" described in the examples is one such nucleotide sequence.

The method of the present invention further relates to a method of altering neurotransmitter metabolism, directly or indirectly, by altering the level of a gene product in a cell. In particular, neurotransmitter metabolism can be altered in neuronal (e.g., striatal neurons) or non-neuronal cells (e.g., glia, fibroblasts). Alterations in metabolism of classical (e.g., GABA, monoamines such as dopamine, norepinephrine) or peptide (e.g., somatostatin, enkephalins, vasoactive intestinal peptide (VIP)) neurotransmitters can occur

presynaptically, postsynaptically, and/or at the level of uptake, for example. Thus, alterations can occur in neurotransmitter biosynthesis, release, uptake, action and/or breakdown, for example. In particular, a nucleotide sequence encoding a gene product which is capable of altering neurotransmitter metabolism, is introduced into a defective HSV-1 vector such that the gene product is functionally expressed from a promoter in the defective HSV-1 vector. The defective HSV-1 vector construct, specifying production of the gene product is packaged with helper virus and introduced into a target cell by infection.

Certain gene products can affect more than one aspect of neurotransmitter metabolism. For example, gene products capable of altering neurotransmitter biosynthesis, can alter neurotransmitter release as well.

In one embodiment of the present invention, a defective HSV-1 vector construct, capable of expressing tyrosine hydroxylase (TH), such as pHSVth, is introduced into a cell. In a non-neuronal cell, such as a fibroblast, the level of tyrosine hydroxylase, which is a rate-limiting enzyme for neurotransmitter biosynthesis, is altered by introduction of the construct, and monoamine neurotransmitter release is altered (increased). Introduction of a defective Herpes virus vector construct, capable of expressing tyrosine hydroxylase (TH), such as pHSVth, into a neuronal cell also results in an increase in monoamine neurotransmitter release.

neurons and glia. In contrast, defective HSV-1 virus vectors are useful for therapy in post-mitotic cells and provide a powerful approach to gene therapy of neurological disorders.

In another embodiment, a gene product that participates in a signal transduction or second messenger pathway (i.e., a signal transduction factor), such as adenylate cyclase, cAMP-dependent protein kinase, protein kinase C, the calcium-calmodulin dependent protein kinase II, or parvalbumin (a calcium binding protein) is selected. Such signal transduction or second messenger pathway gene products, have been implicated in regulating the frequency of action potentials (Madison, D.V. and Nicoll, R.A., J. Physiol. 372: 245-259, 1986)) and neurotransmitter release (Nichols, R.A. et al., Nature 343: 647-651 (1990); Zurgil, N. and Zisapel, N., FEBS Letts. 185: 257-261 (1985)).

The entire gene product or an active or regulatory fragment thereof can be expressed. For example, discrete catalytic and regulatory domains have been recognized in a number of these gene products, such as yeast adenylate cyclase. Other signal transduction enzymes with defined catalytic domains are known, such as cyclic nucleotide phosphodiesterases, both serine/threonine and tyrosine protein kinases and protein phosphatases, phospholipases (e.g., phospholipase C), transcription regulation factors (e.g., the cAMP response element binding protein CREB, fos, jun), and some components of the neurotransmitter release machinery (e.g., synapsins).

The catalytic domains of adenylate cyclase, such as yeast adenylate cyclase (Kataoka, T. et al., Cell 43: 493-505 (1985)), protein kinase C (Mochly-Rosen, D. and Koshland, D.E., J. Biol. Chem. 262: 2291-2297 (1987)), and the calcium-calmodulin dependent protein kinase II (Levine, H. and Sahyoun, N.E., Eur. J. Biochem. 168: 481-486 (1987)) have been isolated and shown to display unregulated activity. Expression of each of the nucleotide sequences corresponding to these gene products, or fragments thereof, from a defective HSV-1 vector can generate an active gene product, capable of altering neurotransmitter metabolism. Expression of the regulatory portion of the molecule could affect neurotransmitter metabolism in the opposite manner. Dominant negative mutant gene products (Herskowitz, I. Nature 329: 219 (1987)) can be used in the present method to interfere with a particular cell function.

In one embodiment, a nucleotide sequence encoding an active catalytic fragment of yeast adenylate cyclase is introduced into a defective HSV-1 virus vector, to make pHSVcyr (Figure 7) such that a functional gene product is expressed when pHSVcyr is introduced into a target cell. Expression of a gene product with adenylate cyclase activity occurs, and, in sympathetic neurons, pHSVcyr caused increases in the cAMP concentration in the cell body, in protein phosphorylation, and in monoamine neurotransmitter release.

In a second embodiment, a nucleotide sequence encoding an active catalytic fragment of a protein

kinase (e.g., a serine/threonine protein kinase) is introduced into a defective HSV-1 virus. For example, a defective HSV-1 vector contruct (pHSVpkcΔ), encoding an active catalytic domain of the rat protein kinase C β-II was made. Expression of the gene product of pHSVpkcΔ was verified, and caused an increase in both monoamine (e.g., dopamine) and excitatory amino acid (aspartate and glutamate) neurotransmitter release in the presence of depolarizing agents. A calcium-dependent effect on neurotransmitter release was observed in both sympathetic and cortical neurons, indicating that neurotransmitter release can be modulated by this method in different types of neurons. In addition, another contruct encoding an active catalytic domain of the calcium/calmodulin dependent protein kinase II, pHSVCaCK, displayed a similar pattern of neurotransmitter release.

In an additional embodiment, a nucleotide sequence which encodes the calcium binding protein parvalbumin is inserted into a defective HSV-1 virus vector, to make pHSVparv, such that a functional gene product is expressed when pHSVparv is introduced into a target cell. As calcium is a second messenger, a calcium binding protein such as parvalbumin participates in a signal transduction or second messenger pathway and is a "signal transduction factor". Parvalbumin, which is primarily localized to rapid firing inhibitory neurons (e.g., GABAergic neurons), was introduced into sympathetic and cortical neurons, cells which do not normally contain parvalbumin. Increases in

monoamine and excitatory amino acid neurotransmitter release were observed in neurons infected with pHSVparv.

Activation of the cAMP pathway by infection with pHSVcyr virus increased neurotransmitter release in the basal state, but not following depolarization. In contrast, activation of either calcium dependent protein kinase pathway by infection with pHSVpkcΔ or pHSVCaCK increased neurotransmitter release following depolarization, but not in the basal state. A third pattern was achieved by expression of parvalbumin, which increased neurotransmitter release in both the basal state and following depolarization. These observations demonstrate that stable activation of signal transduction pathways by introduction of signal transduction factors in defective Herpes virus vectors can cause distinct long term effects on the function of normal neurons. By selecting the appropriate signal transduction factor for introduction into neuronal cells, a different pattern of neurotransmitter release can be attained.

Another class of molecules involved in signal transduction which can be used in the present invention are receptors (e.g., neurotransmitter receptors, growth factor receptors, neurotrophic factor receptors). For example, a number of cell surface receptors for growth factors and neurotrophic factors are protein tyrosine kinases (i.e., tyrosine kinase receptors), which activate signals triggering trophic effects upon binding of the appropriate ligand. Therefore, receptors such

as the EGF receptor, fibroblast growth factor receptors 1 and 2, or neurotrophin receptors TrkA, TrkB and TrkC, represent another kind of "signal transduction factor". Another "signal transduction factor" is the low affinity nerve growth factor receptor, which belongs to a class of receptors which is distinct from the tyrosine kinases.

In one embodiment of the present invention, a nucleotide sequence encoding an unregulated catalytic fragment of a receptor such as the EGF receptor or an FGF receptor is inserted into a defective HSV-1 vector and expressed in neural cells. Expression of an unregulated portion of the receptor with tyrosine kinase activity can activate the signal transduction pathway associated with the selected receptor.

In another embodiment, a nucleotide sequence encoding a version of the low affinity NGF receptor which lacks the extracellular ligand binding domain is inserted into a defective HSV-1 vector to make a construct such as pDB3. The receptor fragment encoded by pDB3 is designed to trigger the effects of its corresponding ligand (e.g., NGF) in the absence of the ligand. This construct can be used to bypass or supplement the requirement of a cell for NGF. For example, cells which do not receive sufficient quantities of target-derived NGF due to injury or disease (e.g., Alzheimer's disease) can be infected with a packaged pDB3 virus construct. Expression and activity of the encoded fragment can mimic the effects of NGF (e.g., maintenance of the phenotype of cholinergic neurons or the

noradrenergic features of sympathetic neurons) even in the absence of NGF. Thus, infection with pDB3 can permit maintenance or restoration of levels of choline acetyl transferase or tyrosine hydroxylase, thereby altering neurotransmitter release. In addition, introduction of pDB3 into a cell could alter the cell so as to express cholinergic or adrenergic features (e.g., induce expression of neurotrasmitter metabolic enzymes such as TH and ChAT) which it did not previously express.

In another embodiment, the GAP-43 targeting sequence is incorporated into the nucleotide sequence of the active catalytic fragment in a defective Herpes virus vector, such that the nucleotide sequence is operably linked to a neuronal cell-specific promoter in the vector. Following packaging with a mutant helper virus, and infection of target cells, the gene product, such as an active fragment of adenylate cyclase, is functionally expressed and targeted to neuronal processes. The effect of the product on neurotransmitter metabolism can be altered by targeting the gene product to a specific region of the cell, where its activity can be enhanced, for example.

pHSVcyr and other HSV-1 vector constructs capable of increasing cAMP levels in a cell, can be used to alter neurotransmitter metabolism at the transcriptional level. The presence of the cAMP response element (CRE) in the regulatory region of the genes for tyrosine hydroxylase and peptide neurotransmitters somatostatin and VIP causes these genes to be transcriptionally activated by cAMP in

neural cell lines (Montimony, M.R. et al., Trends in Neurosci. 13: 184-188 (1990)).

In addition, components of the neurotransmitter machinery, such as synapsin I or synaptophysin, can be selected as gene products for use in altering neurotransmitter metabolism, particularly of neurotransmitter release.

Defective HSV-1 vector constructs, such as pHSVcyr, pHSVpkcΔ, pHSVCaCK, pHSVparv, pHSVngf, and pHSVth, which are capable of altering neurotransmitter metabolism, can be introduced into neural or neuronal cells to alter a disease state. For example, increasing neurotransmitter synthesis or expression of neurotransmitter biosynthetic gene products (e.g., TH, ChAT) can alter a disease process associated with perturbations in neurotransmitter metabolism.

For prophylactic and therapeutic use in altering a disease state (i.e. treatment of a disease) the desired defective HSV-1 virus particles (e.g., pHSVth virus) can be administered in a number of modes, such that the HSV-1 vector or construct is introduced into cells to alter the level of the desired gene product and acts to prevent, reduce, or reverse a disease process. For example, virus can be delivered directly into the organ of interest by injection or into the brain by stereotactic injection (Heuther, G. Stereotactic Neurosurgery, Wilkins, Baltimore (1988)). Alternatively, virus can be delivered into the brain or organ of interest by intravenous or subcutaneous administration,

following incorporation of virus particles into liposomes (Ostrom, M.J., Liposomes: From Biophysics to Therapeutics, Marcel Dekker, New York, (1987)) or polymers (Brown, L. et al., Diabetes 35: 692-697 (1986)).

In treatment of Parksinson's disease, stereotactic injection of pHSVth virus into the striatum, for example, can be used to introduce the pHSVth vector and encoded tyrosine hydroxylase gene into striatal neurons and neurons projecting into the striatum by infection of those cells. In treatment of Alzheimer's disease, a construct which directs the expression of NGF can be introduced into the septal nuclei which contain deteriorating cholinergic neurons or into the pyramidal neurons of the hippocampus, for example, to maintain the cholinergic phenotype of CNS neurons (e.g., basal forebrain cholinergic neurons). The location of the cells infected with the packaged virus contruct is determined by several factors, such as the site of injection, the location of neurons which project to the site of injection, the number of virus particles administered, and the extent of diffusion of the particles.

Other possible methods of delivery of packaged virus to the brain include, but are not necessarily limited to, surgical implantation of a cerebral minipump (Harbough, R. E., Neurosurgery 15: 514-518 (1984)), and transient breach of the blood-brain barrier by osmotic disruption (Neuwelt, E.A., Neurosurgery 7: 44-52 (1980)). The latter method is particularly suitable in cases where delivery throughout the brain is desired. Another possible

mode of administration involves the implanation of genetically modified post-mitotic cells which express the desired gene product from a defective Herpes virus vector. For example, a defective HSV-1 vector can be used to deliver genes into neurons in culture and the cells can subsequently be transplanted into the brain where they will stably express the gene product.

Current therapies for Parkinson's are centered around compensating for the lowered dopamine levels in the striatum. Clinical and basic research efforts include precursor loading by oral administration of L-DOPA (Yahr, M.D. and Bergmann, J. (Eds.), Parkinson's Disease, Raven Press, New York, 1987); Cotzias, G.C. et al., N. Engl. J. Med. 276: 374-379 (1967); Yahr, M.D. et al., Arch. Neurol. 21:343-354 (1969); Rossor, M.N. et al., J. Neurol. Sci. 46: 385-392 (1980); Martin W. E., JAMA 216: 1979-1983 (1971)), dopamine agonists such as bromocryptine (Yahr, M.D. and Bergmann, J. (Eds.), Parkinson's Disease, Raven Press, New York, (1987)), autologous or fetal tissue transplants (Freed, W.J. et al., Ann. Neurol. 8: 510-519 (1987); Lindvall, O. et al., Ann Neurol. 22: 457-468 (1987)) or implantable dopamine delivery systems (Hargraves, R. and Freed, W.J., Life Sci. 40: 959-966 (1987); Freese, A. et al., Exp. Neurol. 103: 234-238 (1989); During, M.J. et al., Ann. Neurol. 25: 351-356 (1989)). Use of defective HSV-1 vectors encoding human tyrosine hydroxylase in the method of the present invention provides a method of gene therapy

for Parkinson's disease, providing an attractive alternative to oral administration of L-DOPA, which loses its effectiveness over time, and to tissue transplantation, which has technical and practical difficulties.

Further, because of the broad host range of HSV-1, the present method is applicable to animal models of disease, such as Parkinson's disease. For example, injection of the neurotoxin 6-OH-dopamine into the substantia nigra of rats results in destruction of dopaminergic neurons which project into the striatum and provides a testable rotational model (Zetterstrom, T. et al., Brain Res. 376: 1-7 (1986)). Alternatively, a primate model of a Parkinsonian syndrome, which is characterized by dopamine depletion in the nigrostriatal system, can be induced by the neurotoxin MPTP (Langston, J.W. et al., Science 219: 979-980 (1983)). Different defective HSV-1 vectors could be assayed using behavioral tests for recovery of dopaminergic function in these animal models. The packaged Herpes virus vectors can be introduced into the brain by stereotactic injection (Pellegrino, L.J., and Cushman, A.J., Methods in Psychobiology, pp. 67-90 (1971); Paxinos, G. and Watson, C., (1986), The Rat Brain in Stereotaxic Coordinates, Second edition, Academic Press, NY). Modification of neural physiology with recombinant defective HSV-1 vectors provides an approach to investigating neurological disorders in model systems, neurotransmitter metabolism, second messenger systems, and gene expression.

For example, the effect of a defective HSV-1 vector construct encoding a signal transduction factor was tested in an assay for apomorphine-induced rotational behavior in rats (Hefti, et al., Pharmacol. Biochem. Behav. 12: 185-188 (1980)). Stereotactic injection of pHSVpkcΔ virus into a rat, led to apomorphine-induced rotational behaviour consistent with an asymmetry of striatal dopamine receptors induced by an increase in neurotransmitter release. The direction of rotation relative to the injection site suggested that pHSVpkcΔ efficiently infected nigral dopamine cells in the right nucleus and that these cells expressed an active catalytic domain of protein kinase C which caused an increase in firing frequency and dopamine release. In response to the increased neurotransmitter release in vivo, post-synaptic dopamine receptors on striatal follower cells were down regulated on the injected side. This receptor asymmetry was reflected in rotational behavior upon the administration of apomorphine. Animals stereotactically injected with the packaged defective HSV-1 vector constructs and deletion helper virus were healthy. This supports the safety and effectiveness of defective HSV-1 vectors in delivering a gene into neural cells of a mammal by the method of the present invention. The vectors can direct expression of a gene inserted into the vector to alter the level of the encoded gene product in target cells, and can alter neurotransmitter metabolism (e.g., neurotransmitter release) in the mammal.

The invention is further and more specifically described in the following examples which are not intended to be limiting in any way.

Example 1

pHSVth Directs Expression of Active TH in Fibroblasts

Construction of pHSVth

A full length human tyrosine hydroxylase (TH) cDNA (HTH-2) was isolated from a human neuroblastoma cDNA library (Ginns, E.I. et al., Soc. Neurosci. Abstr. 13: 859 (1987); O'Malley, K.L. et al., Biochemistry 26: 6910-6914 (1987)), using a rat TH cDNA as a probe (O'Malley, K.L., J. Neurosci. Res. 16: 3-13 (1986)). The cDNA was inserted into a variety of vectors, including pHSV1ac (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988)). In order to clone the human TH gene into pHSV1ac, the vector was digested to completion with Hind III and then partially digested with Eco RI, removing the lacZ coding sequence. A Hind III-Eco RI fragment of the human TH gene was subcloned into pHSV in a unidirectional fashion using the available Hind III and Eco RI sites to make pHSVth (Figure 6). Verification of the orientation of the fragment with respect to the IE 4/5 promoter (McGeoch, D.J. et al., Nucleic Acids Res. 14: 1727-1745 (1986)), and the integrity of the reading frame and the translational start site was confirmed by sequencing using TH specific oligonucleotides.

Packaging of pHSVth

pHSVth DNA was packaged into HSV-1 virus particles by the method of Geller, using HSV-1 strain 17 ts K as helper virus. (Geller, A.I., Nucleic Acids Res. 16: 5690 (1988)). Briefly, 1 X 10^5 CV1 cells were seeded on a 60-mm plate. The following day the cells were transfected (Graham, F.K., Virology 52: 456-467 (1973)) with a 0.5-ml calcium phosphate coprecipitate containing 1 μg of pHSVth DNA and 9μg of salmon sperm DNA. Four hours later, the cells were treated with 15% glycerol (Parker, B.S. and Stark, G.R., J. Virol. 31: 360-369 (1979)). Following a 24 hour incubation at 37 ^{o}C, 1.5 X 10^6 plaque-forming units (pfu) of HSV-1 ts K (Davison, M.J. et al., J. Gen. Virol. 65: 859-863 (1984)) in 100 μl of medium was added to each plate. After 1 hour at room temperature, an additional 5 ml of medium was added to each plate. Following an incubation of 3 days at 31 ^{o}C, virus was harvested. Virus was passaged at a 1:2 dilution on CV1 cells at 31 ^{o} C. Virus was prepared, passaged, and titered as described (Miller, R.H. and Hyman, R.W. Virology 87: 34-41 (1978)).

Infection of CV1 cells and Analysis of Viral DNA

1 X 10^7 CV1 monkey fibroblasts were infected with 5 X 10^7 infectious particles of pHSVth virus and the cells were incubated for 24 hours at 31 ^{o}C. The presence of viral DNA was ascertained by amplification via the polymerase chain reaction.

Oligonucleotide primers OhTH-116 and OhTH-193 were derived from the human TH cDNA sequence (HTH-1) and were synthesized by the Protein Chemistry Facility (Washington University). Oligonucleotide OhTH-116 (5'-dGGGCTTCCGCAGGGCCGTGTCTGAGCTGGA) is identical to the coding sequence of nucleotides 36-65, and OhTH-193 (5'-dAGGGACTGCAGCGGCCGCTGCTGCCACC) is complementary to the coding sequence of nucleotides 156-185 of HTH-1(Grima, B. et al., Nature 326: 707-711 (1987)).

Control and infected cells were lysed in a PCR-compatible buffer containing nonionic detergents and proteinase K. After incubation at 60 °C for one hour, the proteinase K was inactivated by heating and an aliquot of the mixture was added to an amplification reaction. ^{32}P-end labeled primers from exons 1 and 2 (OhTH-116, OhTH-193) of the human TH gene were used to amplify TH cDNA in the mixture. An aliquot of the PCR reaction mixture was electrophoresed on a 5% acrylamide gel. The gel was dried and subjected to autoradiography to identify the diagnostic TH band.

The predicted size of the PCR fragment obtained with primers OhTH-116 and OhTH-193 is 161 base pairs. A band of the expected size, as compared to the corresponding human TH cDNA fragment, was detected in the PCR products from pHSVth infected fibroblasts. In contrast, no band was observed in the PCR products of human genomic DNA, mock infected fibroblasts, or HSV-1 ts K infected fibroblasts.

Detection of TH by Immunofluorescence

pHSVtk infected CV1 cells (multiplicity of infection of 0.1) were assayed for TH immunoreactivity one day post-infection. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, and immunohistochemistry was performed as described (Huettner, J.E. and Baughman, R.W., J. Neurosci. 6: 3044 (1986), using commercially available mouse anti-human TH (Boehringer Mannheim Biochemicals, #1017-381) as the primary antibody. Forty μg of anti-TH antibody was reconstituted in 1.0 ml of double distilled water, and was further diluted 1:10. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ diluted 1:200. Coverslips were mounted in phophate buffered saline and glycerol (1:1) containing 0.4% n-propyl gallate.

TH immunoreactivity was observed in approximately 10% of the cells. Because of the low multiplicity of infection, TH-positive cells are easily detected in the background of surrounding negative cells. In addition, uninfected cells were negative for staining.

Determination of TH Activity

A nonenzymatic coupled decarboxylation assay was used to measure TH activity in infected fibroblasts one day post-infection (Waymire, J.C. et al., Anal. Biochem. 43: 588-600 (1971)). Frozen cell pellets were lysed in 0.2% Triton X-100 and protease inhibitors. Protein concentrations were

determined by the method of Lowry (Lowry, O.H. et al., J. Biol. Chem. 193: 265-275 (1951)). Table 1 shows that the TH activity of mock infected cells or cells infected with helper virus alone (HSV-1 ts K) was negligible, while pHSVth infected cells exhibited a level of TH activity (pmoles DOPA/μg protein/hr) approximately 40% that of rat striatal cells assayed in parallel.

These results demonstrate that pHSVth was faithfully packaged into virus particles using the HSV-1 strain 17 ts K as helper virus. The packaged pHSVth was able to infect fibroblasts, and to direct production of active TH in these cells as monitored by immunofluorescence and enzymatic assays.

TABLE 1

Tyrosine Hydroxylase Activity in pHSVth Infected CV1 Fibroblasts

Cell Type	pmoles/ DOPA/μg protein/hr
mock infected CV1	0.002 ± 0.004
HSV-1 ts K	0.002 ± 0.003
pHSVth	0.181 ± 0.025
rat striatum	0.457 ± 0.063

Example 2
pHSVth Directs Expression of TH in Rat Striatal Cells

Primary cultures of rat striatal neurons were prepared as described (Freese, A. et al., Brain Res., in press (1990)). Cultures were prepared on five 13-mm glass coverslips in a 35-mm dish: each glass coverslip was coated with 20μg of poly(L-lysine). Five days after plating, cultures were treated with 40 μM arabinonucleoside (cytosine arabinoside) for 24 hours to prevent glial overgrowth. Five to sixteen days after the arabinonucleoside treatment, cultures were infected with the same pHSVth virus stock described in Example 1. Each 35-mm dish contained about 1×10^5 cells at the time of infection, and about 10% of the cells were neurons.

In order to determine if TH was expressed in neurons, immunofluorescence studies were performed to determine if TH and a neuronal marker (neurofilament) were present in the same cells. At times two days post-infection and one week post-infection, cells were fixed and processed for immunohistochemistry as described in Example 1. Mouse antibody specific for TH (Boeringer Mannheim Biochemicals #1017-381) and an IgG fraction of rabbit anti-neurofilament-200 (Sigma Chem. Corp., St. Louis, Mo., #N-4142) were used as primary antibodies. Secondary antibodies were fluorescein isothiocyanate-conjugated goat $F(ab')_2$ antibody

against mouse $F(ab')_2$ (1:200 dilution) and rhodamine isothiocyanate-conjugated goat $F(ab')_2$ antibody to rabbit $F(ab')_2$ (1:25) dilution.

The analysis revealed colocalization of TH and neurofilament immunoreactivity, indicating that pHSVth directed expression of TH in neuronal cells. 10% of the total cells showed NF immunoreactivity, identifying the neuronal cells. Consistent with the multiplicity of infection, 10% of the neurons, and 10% of the glia showed TH immunoreactivity.

Example 3
Neurotransmitter Release from pHSVth Infected Fibroblasts and Striatal Neurons

Cell Culture Release Experiments

CV1 fibroblasts were maintained and infected as described in Example 1. Primary cultures of 1 to 4-day-old rat striatal neurons were prepared and infected as described in Example 2. Cultures were infected with pHSVth, pHSVpUC, HSV-1 ts K virus, or were mock infected. HSV-1 strain 17 ts K was used as helper virus in packaging. pHSVpUC is a derivative of pHSVlac (*c.f.*, Example 1), in which the *Eco RI-Hind III* fragment encoding *lacZ* of pHSVlac was replaced by the polylinker of pUC19 to make pHSVpUC.

Two to three days or one week post-infection, the cell culture medium was removed and the cells were washed in release buffer and then incubated in 200μl of the release buffer for 15 minutes. This release buffer was then aspirated off the cells,

cooled on ice water (in prechilled tubes), and centrifuged for 5 minutes (1400 rpm) to remove any cellular debris. 20μl of 2M $HClO_4$ and 20μl of 1% $Na_2S_2O_5$ were added, and the samples stored at -70°C until analysis by HPLC. The release buffer was of the following composition: 135mM NaCl, 3mM KCl, 1mM $MgCl_2$, 1.2mM $CaCl_2$, 2mM $NaPO_4$, 200μM ascorbate and 10μM glucose. The following drugs were added to the release buffer where indicated: tyrosine (1mM), the tyrosine hydroxylase co-factor, tetrahydrobiopterin (BH_4, 1mM), tetrodotoxin (1μM), veratridine (5mM), calcium-free, where 0.1 mM EGTA replaced the calcium and high potassium buffer to depolarize cells where the KCl was increased to 56mM and NaCl reduced to 80mM to maintain osmolarity.

HPLC Analysis

Samples were thawed and spun through a 10,000 Dalton regenerated cellulose filter prior to direct HPLC injection. The direct injection technique was compared with a standard acid-washed alumina extraction method. Sample analysis was performed using HPLC with a series array of 16 coulometric electrode sensors (CEAS, Model 55-0650, ESA, Inc. Bedford, MA) (Matson, W. R. et al., Clin. Chem. 30:1477-1488 (1984); Matson, W. R. et al., Life Sci., 41:905-908 (1987)). The basic principle of this system is that the 16 electrodes set at incremental potentials provide an additional dimension of separation of the eluting compounds. As the coulometric electrodes fully oxidize at 100%

for a given potential, the following sensors are essentially independent. This allows a compound to be defined not only by its elution time, but also by its specific oxidation pattern. For example, at 60 millivolt (mM) incremental settings, dopamine has a dominant response on electrode 2 (60mV versus a palladium reference electrode), the response on detector 1 (0mV) or detector 3 (120mV) is only 10-30% of its response on the dominant 60mV electrode. A specific peak ratio is characteristic for any given compound and coupled with reproducible, defined elution time enables great precision and specificity for determining a peak's authenticity as any coeluting compound is likely to alter the peak ratio.

A gradient method which had been optimized by Dr. I. N. Acworth of ESA, Inc. was used for the resolution of catecholamines. Two mobile phases were used, "A" was 0.1M NaH_2PO_4 with 10mg/L of dodecyl sulfonic acid, and 100nM nitrilotriacetic acid, adjusted to pH 3.45 with phosphoric acid; the B mobile phase was 0.1M NaH_2PO_4 (pH 3.35) with 50 mg/L of dodecylsulfonic acid and 100nM nitriloacetic acid, 50% methanol v/v. Several different electrode settings were used. The primary potential settings were 60mV increments from 0 to 900mV, in addition, a 50mV incremental system was used with electrodes 1 to 4 set at increments from 50 to 250mV. Finally, for selected samples a gate-cell array was used where the electrodes were set at oxidizing potentials alternating with reducing (negative)

potentials - the concept behind this system is that only those compounds which reversibly oxidize and reduce at the defined potentials will pass the "gate" (Matson et al., Clin. Chem. 30: 1477-1488,1984). An 8 by 0.45 cm, 3μm C18 ESA HR80 (Teflon) column was used for the majority of assays with a 15 cm by 0.45cm, 5μm Nikko Bioscience (Tokyo, Japan) column used to help improve resolution of norepinephrine. The gradient was set to ramp mobile phase B from 6% at time 0 to 40% at 15 minutes, a further gradient up to 90% B at 23 minutes with an 11 minute re-equilibration period. Chromatograms were completed within 30 minutes and injections were set using an ESA refrigerated Model 460 autoinjector at 35 minute intervals.

The validity of detected peaks was determined by comparison with standards for elution time and ratio accuracy and then quantitated by relative peak height on the dominant sensor.

CV1 Fibroblasts Infected with pHSVth Virus Release L-Dopa and Dopamine

CV1 fibroblasts infected with HSV-1 ts K virus alone, yielded 0.17 pg/μl L-Dopa in the release buffer, while CV1 cells infected with pHSVth virus stock yielded 0.58 pg/μl L-Dopa. The elevation in L-Dopa production is consistent with the observation of TH-immunoreactivity and elevated TH enzyme activity observed in pHSVth virus infected cells.

Dopamine production was also measured. 0.08 pg/μl of dopamine was detected in release buffer

from mock infected CV1 cells, and 0.06 pg/μl of dopamine was detected in release buffer of HSV-1 ts K virus infected cells. In contrast, 0.56 pg/μl of dopamine was detected in release buffer from pHSVth virus infected CV1 cells.

Primary Neonatal Rat Striatal Cultures Infected with pHSVth Virus Release L-Dopa

Basal levels of L-dopa were detected in the release buffer of all pHSVth infected cells at one week. Levels were 0.098± 0.005 pg/μl in those cells treated with BH_4 co-factor alone and increased to 0.395± 0.003 pg/μl in those cells treated with both cofactor and tyrosine. Depolarizing the cells with 56mM K^+ increased L-dopa release to 0.170± 0.005 pg/μl in the cofactor alone group, with levels increasing to 0.683 ± 0.005 pg/μl in the cofactor plus tryosine group. The neuronal cells in the striatal culture are responsible for the K^+-dependent component of L-Dopa release, as glia would not be expected to show K^+-dependent release.

The release buffer from cells infected with pHSVpUC or mock (untreated) cells shows some chromatographic peaks consistent with L-dopa. The levels were however variable and were lower on successive washings of the cells, with no increase in signal on K^+ induced depolarization. This suggested that the L-dopa from these cells was more likely a contaminant from the growth medium which includes horse serum, a significant source of L-dopa as demonstrated by HPLC analysis.

Dopamine Release

Basal levels of dopamine were not detected in any cells infected with pHSVpUC or mock-treated with cofactor alone also had undetectable levels. However, the pHSVth cells treated with both cofactor and tyrosine produced dopamine under the high K+ conditions (56mM) with levels of 0.028 pg/μl in the release medium. This result suggests the presence of the enzyme aromatic amino acid decarboxylase (AAAD) in addition to tyrosine hydroxylase in the infected cells.

Example 4

The following materials and methods were used in Examples 5-11. The data in Examples 5-11 demonstrate that specific activation of the cAMP signal transduction pathway by expression of a fragment of yeast adenylate cyclase from a defective HSV-1 virus vector, pHSVcyr, stably alters the physiology of both undifferentiated PC12 cells and cultured rat sympathetic neurons. One day after infection of PC12 cells, pHSVcyr increased cAMP levels, protein phosphorylation, and neurotransmitter release. In sympathetic neurons, pHSVcyr caused increases in the cAMP concentration in the cell body, in protein phosphorylation, and in monoamine neurotransmitter release.

Construction of pHSVcyr, pHSVpUC, and Packaging Into HSV-1 Particles

pHSVcyr was constructed from pHSVlac using standard recombinant DNA techniques (Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY, 1982). The 3.3 kb Hind III-Eco RI fragment containing the Lac Z gene was excised from pHSVlac and replaced with the Hind III-Eco RI fragment containing the pUC19 polylinker, thereby creating pHSVpUC. A 1.5 kb Pvu II-Bal I fragment containing the catalytic domain of the yeast adenylate cyclase (cyr) gene (nucleotides 5465 to 6968; Kataoka et al., Cell 43: 493-505 (1985); provided by Dr. Wigler) was isolated. Hind III linkers were ligated to the fragment, and it was cloned into the unique Hind III site of pHSVpUC. HSV-1 vectors (e.g. pHSVpUC and pHSVcyr) were packaged into HSV-1 particles as described (Geller, A.I., Nucleic Acids Res. 16: 5690, 1988), using HSV-1 strain 17 ts K as helper virus. The titer of the virus stock was 2 X 10^6 plaque forming units (pfu)/ml ts K and 9 X 10^5 infectious particles/ml pHSVcyr.

Cell Culture and Virus Infections

CV1 monkey fibroblasts were grown in Dulbecco's modified minimum essential medium (DMEM) with 10% fetal bovine serum. PC12 rat pheochromocytoma cells (Greene, L.A. and Tischler, A.S., Proc. Natl. Acad. Sci. USA 73: 2424-2428, 1976) were grown in RPMI 1640 containing 10% horse serum and 5% fetal bovine

serum. 0.5 ml PC12 cells ($1X10^4$ cells/ml) were plated in 24 well plates, when the cell density reached $2x10^5$ cells/0.5 ml, the cultures were infected with pHSVcyr (7.5 μl) or pHSVpUC (7.5 μl); and one day later assays were performed.

Cultures of superior cervical ganglia were prepared as described (Hawrot, E. and Patterson, P.H., Methods in Enzymology 58: 574-588, 1979) from 1 to 4 day old Sprague Dawley rats (nerve growth factor was provided by Dr. Johnson; all other tissue culture reagents were obtained from GIBCO). 24 well plates were pretreated for 3 hours with 300 μl of 0.1 mg/ml collagen (cal biochem), in 1% acetic acid and washed two times with 0.5 ml PBS before use; five days after plating cultures were treated with 40 μM cytosine arabinoside for 24 hours to prevent glial overgrowth. Five to sixteen days after the cytosine arabinoside treatment cultures were infected with pHSVcyr (7.5 μl) or pHSVpUC virus (7.5 μl); six days later parallel wells were infected and one day later assays were performed (seven days and one day after infection, respectively). Each well in a 24 well plate contained approximately $2x10^5$ cells in 0.5 ml at the time of infection and approximately 20% of the cells were neurons. For in situ hybridization or immunofluorescence, both PC12 cells and superior cervical ganglia were plated on 12 mm glass coverslips (in 24 well plates), coated with collagen as described above.

DNA Analysis

$1X10^7$ CV1 cells were infected with $5x10^7$ infectious particles of pHSVcyr virus and the cells were incubated for 24 hours at 31°C. Total cellular DNA was isolated (Wigler et al., Cell 16: 777-785, 1979), 5 μg of DNA was incubated with 12.5 units of Eco RI overnight at 37°C, resolved on 0.7% agarose gels, and transferred to Genetran (Plasco Co.) as described (Southern, E.M., J. Molec. Biol. 98:503-517, 1975). The resulting blot was probed with the 3.3 kb Kpn I-Hind III fragment from the plasmid pHSV1ac containing the pBR and HSV c region segments, radiolabeled with ^{32}p (Fineberg and Vogelstein, Anal. Biochem., 132: 6-13, 1983). Hybridization and washing were performed as described (Southern, E.M., J. Molec. Biol. 98:503-517, 1975).

RNA Analysis Using the Polymerase Chain Reaction

$2x10^7$ CV1 cells were infected with $2x10^6$ infectious particles of pHSVcyr or pHSVpUC virus, and incubated for 24 hours at 37°C. Total cellular RNA was prepared as described (Chirgwin et al., Biochemistry 18: 5294-5299, 1979). 1 μg of each RNA sample was suspended in 24 μl H_2O and treated with 6 μl 0.01 M MeHgOH followed by 3.1 μl 0.7 M 2-mercaptoethanol to remove RNA secondary structure, and cDNA was synthesized from each section in a 50 μl in situ transcription reaction (50 mM Tris pH 8.2 at 42°C, 50 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, 1000 u/ml Promega Biotech RNAsin, 1 ug 3' primer, 5 mM DNTPs, 1000 u/ml Life Sciences AMV reverse transcriptase)

at 42°C for 2 hours. The reaction was placed at 65°C for 10 minutes to inactivate the reverse transcriptase, 2 μl of each reaction was transferred to a 100 μl polymerase chain reaction (PCR) mix (Perkin Elmer Gene Amp kit). The cDNA was subjected to 40 cycles of PCR (94°C, 1 min; 60°C, 2 min; 72°C, 3 min). 40 μl of each reaction was electrophoresed on a 1% agarose/1% Nusieve gel in tris acetate EDTA buffer and transferred to Hybond (Amersham) in 20xSSC, following which the filter was hybridized to a cyr cDNA probe. The 3' primer used for both cDNA synthesis and the PCR reaction was antisense to a portion of the SV40 polyadenylation sequence immediately 3' from the cyr gene; its sequence is 5'CGTTCGTGCCTTCCCGCAGGAGGAACGTCC3'. The 5' primer used in the PCR reaction was a 27 base oligonucleotide from the cyr gene nucleotides 5688 to 5714 (Kataoka et al., Cell 43: 493-505, 1985); its sequence is 5'GGCTTACGGCTGTAGAGAGAATATTA3'. The size of the predicted product from the PCR reaction is 1500 bp.

In Situ Hybridization

Cells on coverslips were fixed for 15 minutes in 4% paraformaldehyde in 0.1 M NaPO4 pH 7.0, then rinsed with 70% ethanol and stored in 70% ethanol at 4°C until processed for in situ hybridization. Cells were rehydrated in PBS for 2 minutes, pre-treated in 20 mM HCl, 0.01% Triton X-100, and 1 ug/ml proteinase K, postfixed with 4% paraformal-dehyde, and acetylated by immersing the slides in

100 mM triethanolamine, pH 8.0, 0.25% acetic anhydride, and stirring for 10 minutes. After the cells were rinsed in PBS with 2 mg/ml glycine, then were prehybridized in 50% deionized formamide, 2X SSC, 25 μg/ml yeast tRNA, 250 μg/ml salmon testes DNA, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.2% SDS, 25 mM EDTA, 10% dextran sulfate for one to several hours at 42°C. The cyr DNA was labeled by random-primer nick translation with ^{35}S dCTP (Amersham > 1000 Ci/mmol). The prehybridization mix was drained from the slides, which were then incubated with hybridization buffer (prehybridization buffer which contained 2X 10^6 cpm/ml of hybridization probe) at 42°C overnight. After hybridization, sections were washed in 2X SSC, 1X SSC and then 0.1X SSC for 30 minutes each at room temperature, then in 0.1XSSC at 42°C and then 55°C. The coverslips were air dried, dipped in Kodak NTB2 emulsion, and exposed at 4°C for two weeks. Slides were developed in Kodak D10 developer and fixed in Kodak fix; the cells were lightly counterstained with 0.1% cresyl violet. Hybridization was observed using brightfield microscopy, and photographed with Tmax 400 film. Cells were fixed with 4% paraformaldehyde in 0.1 M $NaPO_4$ pH 7.0. Immunohisotchemistry was performed as described (Heuttner, J.E. and Baughman, R.W., J. Neurosci. 6: 3044-3060, 1986); the primary antibodies were rabbit anti-yeast adenylate cyclase (Heideman, W. et al., J. Cell Biochem. 42: 229-242, 1990; provided by Dr. Heideman; U1 used at a 1:50

dilution) (ovalbumin) or a rabbit anti-cAMP (1:50 dilution; Chemicon) and monoclonal mouse anti-rat neurofilament (1:800 dilution; SMI-33, Cappel). (This antibody reacts with mouse, rat and human neurofilaments.) The rabbit anti-cAMP antibody was preabsorbed with 1.0 mM cAMP for 30 minutes at 4 °C. The secondary antibodies were fluorescein isothiocyanate-conjugated goat $F(ab')_2$ antibody to mouse $F(ab')_2$ antibody (1:200 dilution, Cappel) and rhodamine isothiocyanate-conjugated goat $F(ab')_2$ antibody to rabbit $F(ab')_2$ (1:250 dilution; Cappel). Coverslips were mounted in PBS glycerol 1:1 containing 0.4% n-propyl gallate.

cAMP Radioimmunoassay

PC12 cells were cultured and infected (moi 0.1) as described above, except 60 mm plates containing 5 ml of medium were used. One day after infection, the media was removed (all subsequent manipulations and solutions were at 4°C), and the cells were washed once with 2 ml of PBS. The cells were lysed in 1 ml of 5% TCA 0.1 M HCl by shaking for 5 minutes. The cell lysates were centrifuged (1400 rpm) for 5 minutes, the supernatants were frozen in dry ice-ethanol, and stored at -70°C. The samples were extracted five time with diethyl-ether, lyophilized, and immediately assayed for cAMP (^3H cAMP assay kit, Amersham) following the protocol provided by Amersham. Each experimental condition (pHSVcyr, pHSVpUC, mock) in an experiment was done

in triplicate, and the experiment was repeated three times.

In vivo Protein Phosphorylation

The media was removed, and the cells were incubated for 30 minutes in 0.3 ml of ^{32}P PO_4 medium (PC12 cells: 2.4 ml DMEM-PO_4 (source), 0.4 ml dialyzed horse serum, .02 ml dialized fetal bovine serum, and 1 ml ^{32}P PO_4 (2 mCi, 8500 Ci/mmol, New England Nuclear); Neurons; 2.6 ml DMEM-PO_4 0.4 ml dialyzed fetal bovine serum, and 1 ml ^{32}P PO_4). Where indicated forskolin (1mM) or bt_2cAMP (2mM) were added. The ^{32}P PO_4 medium was removed, and the cells were lysed in 90 µl lysis buffer by shaking at 0°C for 30 minutes. Lysis buffer contained 100 mM $NaPO_4$ pH 7.0, 50 mM KF, 20 mM EDTA, 5% TX-100, and protease inhibitors (1 uM pepstatin A, 1 mM 1,10-phenanthroline, 0.1 mM PMSF, 1 mM iodoacetamide, 1 µg/ml aprotinin, and 1 µg/ml leupeptin). The lysis buffer was removed, centrifuged at 1400 rpm for 5 minutes, and the supernatant was analyzed by TCA precipitation and SDS polyacrylamide gel (8% acrylamide) electrophoresis (Laemmli, U.K., Nature 227: 680-685, 1970). Gels were treated with enhance (New England Nuclear), dried, and exposed to film (XR-5) for 30 minutes.

Neurotransmitter Release Assays

The cell culture medium was removed, cells were washed once in release buffer, and then incubated in 200 µl release buffer for 15 minutes. The release

buffer was then aspirated off the cells, cooled in ice water for 5 minutes (in prechilled tubes), and centrifuged for 5 minutes (1400 rpm) to remove any cellular debris. 20 μl of 2 M $HClO_4$ and 20 μl 1% $Na_2S_2O_5$ were added, and the samples were stored at -70°C prior to analysis by HPLC. Release buffer: 135 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2 mM $NaPO_4$ pH7.4, 200μm ascorbate and 10 μM Glucose. The following drugs were added to the release buffer where indicated: Forskolin (1 mM), bt_2cAMP (2 mM), tetrodotoxin (1 μM), and veratadine (5 mM). Release buffer without calcium contained 0.1 mM EGTA in place of the $CaCl_2$. (When this release buffer was used, the wash before incubation in release buffer was also performed with this buffer.) Release buffer to depolarize cells contained 56 mM KCl, 80 mM NaCl, and the other components of release buffer.

Sample analysis was performed using HPLC with a series array of 16 coulometric electrode sensors (CEAS, Model 55-0650, ESA, Inc., Bedford, MA; Matson, W.R. et al., Clin. Chem. 30: 1477-1488, 1984; Matson, W.R. et al., Life Sci. 41: 905-908, 1987). The basic principle of this system is that the 16 electrodes set at incremental potentials provide an additional dimension of separation of the eluting compounds. As the coulometric electrodes fully oxidize at 100% for a given potential, the following sensors are essentially independent. This allows a compound to be defined not only by its elution time, but also by its specific oxidation pattern. For example, at 60 millivolt (mV)

incremental settings, dopamine has a dominant response on electrode 2 (60 mV versus a palladium reference electrode), the response on detector 1 (0 mV) or detector 3 (120 mV) is only 10-30% of its response on the dominant 60 mV electrode. A specific peak ratio is characteristic for any given compound and coupled with reproducible, defined elution time enables great precision and specificity for determining a peak's authenticity as any coeluting compound is likely to alter the peak ratio.

A gradient method which had been optimized by Dr. I. N. Acworth of ESA Inc. was used for the resolution of catecholamines. Two mobile phases were used, "A" was 0.1 M NaH_2PO_4 with 10 mg/L of dodecycl sulfonic acid, and 0.1 μM nitrilotriacetic acid, adjusted to pH 3.35 with H_3PO_4; the "B" mobile phase was 0.1 M NaH_2PO_4, pH 3.35) with 50 mg/L of dodecysulfonic acid, and 0.1 μM nitrilotriacetic acid, 50% methanol (vol./vol.). Several different electrode settings were used. The primary potential settings were 60 mV increments from 0 to 900 mV, in addition, a 50 mV incremental system was used with electrodes 1 to 4 set at increments from 50 to 250 mV. Finally for selected samples, a gate-cell array was used where the electrodes were set at oxidizing potentials alternating with reducing (negative) potentials. The concept behind this system is that only those compounds which reversibly oxidize and reduce at the defined potentials will pass the "gate". An 8 cm by 0.45 cm, 3μm C18 ESA HR80

(Teflon) column was used for the majority of assays with a 15 cm by 0.45 cm, 5μm Nikko Bioscience (Tokyo, Japan) column used to help improve resolution of norepinephrine. The gradient was set to ramp mobile phase B from 6% at time 0 to 40% at 15 minutes, a further gradient up to 90% B at 21 minutes with an 11 minute re-equilibration period. Chromatograms were completed within 30 minutes and injections were set using an ESA refrigerated Model 460 autoinjector at 35 minute intervals.

The validity of detected peaks was determined by comparison with standards for elution time and ratio accuracy and then quantitated by relative peak height on the dominant sensor.

Example 5
pHSVcyr virus expresses cyr RNA and protein in PC12 cells

We inserted the portion of the gene encoding the catalytic domain of yeast adenylate cyclase (cyr) into a defective HSV-1 vector to yield the vector pHSVcyr (Figure 7). A vector containing the pUC19 polylinker (pHSVpUC) in place of the cyr gene was used as a control. pHSVcyr and PHSVpUC were packaged into HSV-1 particles using established procedures (Geller, A.I., Nucleic Acids Res. 16:5690, 1988). To determine if pHSVcyr was properly packaged into HSV-1 particles, DNA was prepared from pHSVcyr virus stock and subjected to Southern analysis. The results demonstrated that

pHSVcyr DNA was properly packaged into HSV-1 particles. pHSVpUC DNA was also properly packaged into HSV-1 particles (not shown).

The ability of pHSVcyr to express cyr RNA was examined. CV1 cells were infected with pHSVcyr virus, pHSVpUC virus or mock infected, and one day later total cellular RNA was isolated. cyr cDNA was synthesized using reverse transcriptase and a primer homologous to the 3' end of the cyr transcript, the cyr products were amplified using the polymerase chain reaction with primers homologous to the cyr transcript, and displayed on a polyacrylamide gel. pHSVcyr, but not pHSVpUC or mock infected cells, contained the expected 1.5kb band. Furthermore, pHSVlac expresses an RNA of the predicted size (shown by Northern analysis), consistent with the proper functioning of the transcription regulatory elements in the vector. To show that cyr RNA was expressed in PC12 cells, in situ hybridization was performed. PC12 cells (Greene and Tischler, PNAS 73: 2424-2428, 1976) were infected with pHSVcyr virus, and one day later fixed with 4% paraformaldehyde and hybridized in situ with a probe homologous to the cyr gene. Cultures infected with pHSVcyr virus contained cells which labeled heavily with the probe, whereas cultures infected with pHSVpUC virus, or mock infected cultures, lacked labeled cells.

The ability of pHSVcyr to express cyr protein was examined. PC12 cells were infected with pHSVcyr virus, after twenty-four hours fixed with 4%

paraformaldehyde, and subjected to immunohistochemistry with a rabbit anti-cyr antibody which was raised against a polypeptide from the cyr catalytic domain. The antibody was visualized with a rhodamine conjugated goat anti-rabbit IgG antibody. Cultures infected with pHSVcyr virus contained cells with prominent cyr immunoreactivity (cyr-IR). Cultures infected with pHSVpUC virus, or mock infected cultures, lacked cells with cyr-IR. Cultures infected with pHSVcyr virus and subjected to immunohistochemistry using preimmune rabbit serum lacked cells with rhodamine fluoresence. The results demonstrate that pHSVcyr DNA was properly packaged into HSV-1 particles and that pHSVcyr virus expresses cyr RNA and cyr protein in PC12 cells.

Example 6
pHSVcyr Virus Causes an Increase in cAMP Concentration in PC12 Cells

If the cyr catalytic domain exhibited unregulated adenylate cyclase activity in PC12 cells, then cAMP levels would be elevated. PC12 cells were infected with pHSVcyr virus, one day later a TCA extract was prepared, and the amount of cAMP was determined using a radioimmunoassay for cAMP. Cultures infected with pHSVcyr virus contained two-fold higher levels of cAMP than cultures infected with pHSVpUC, or mock infected cultures. Approximately ten percent of the cells were infected with pHSVcyr, as determined with both

the in situ hybridization assay for cyr RNA and the immunofluorescent assay for cyr-IR; therefore, pHSVcyr caused about a 20X increase in cAMP concentration per infected cell.

An immunofluorescent assay for cAMP was used to confirm that the increase in cAMP was localized to individual cells. PC12 cells were infected with pHSVcyr virus, one day later fixed with 4% paraformaldehyde, and subjected to immunohistochemistry with a rabbit anti-cAMP antibody. The antibody was visualized with the rhodamine conjugated goat anti-rabbit IgG antibody (same secondary antibody used to assay cyr-IR). PC12 cultures infected with pHSVcyr virus contained cells with dramatically elevated levels of cAMP-immunoreactivity (cAMP-IR); longer exposures detected low levels of background cAMP-IR present in all cells. Cultures infected with pHSVpUC virus or mock infected cultures lacked cells with elevated levels of cAMP-IR and cultures infected with pHSVcyr virus and subjected to immunohistochemistry using rabbit anti-cAMP antibody (preabsorbed with cAMP) lacked cells with cAMP-IR. Moreover, cultures infected with pHSVcyr virus and subjected to immunohistochemistry using rabbit serum did not contain any rhodamine fluoresence. (Since cyr-IR and cAMP-IR assays were performed in parallel and used the same secondary antibody, the pHSVcyr infected culture assayed with preimmune rabbit serum served as negative control for both experiments). Treatment of PC12 cells for one day with bt_2cAMP

does not induce neurite outgrowth or differentiation, although short processes are observed after several days (Kalman, D. et al. Neuron 2: 355-366, 1990). Similarly, one day after pHSVcyr infection of PC12 cells, no neurite outgrowth was observed on the cyr-IR cells.

Parallel cultures of PC12 cells infected with the same amount of pHSVcyr virus (moi. 0.1) produced similar numbers of cyr RNA containing cells (in situ hybridization), cyr-IR cells, and cAMP-IR cells (approximately 10% of each), suggesting that the cells expressing the cyr RNA and protein produced the increased cAMP levels. However, it was not possible to colocalize the cyr-IR and the cAMP-IR to the same cell since the antibodies directed against cyr and cAMP were both raised in rabbits. Thus, pHSVcyr directs the synthesis of biologically active adenylate cyclase in PC12 cells which increases cAMP levels approximately 20 fold per infected cell.

Example 7
pHSVcyr Virus Increased Protein Phosphorylation in PC12 Cells

The increase in cAMP levels caused by pHSVcyr was likely to affect specific aspects of neuronal function. Since cultures of neurons contain a heterogenous mixture of different cell types including both neurons and glia, the effects of pHSVcyr on the physiology of PC12 cells, a homogenous cell line with neuronal properties, were

examined. The increase in cAMP levels in PC12 cells infected with pHSVcyr might activate the A kinase, thereby increasing protein phosphorylation.

PC12 cells were infected with pHSVcyr virus, pHSVpUC virus, or mock infected; one day later, these cells were incubated for 30 minutes with ^{32}P PO_4, and protein extracts were prepared (Rossie, S. and Catterall, W.A., J. Biol. Chem. 262: 12735-12744, (1987)). To measure total protein kinase activity in these cells, the amount of ^{32}P PO_4 incorporated into protein was determined by measuring the amount of ^{32}P PO_4 that was precipated by TCA from the protein extracts. pHSVcyr and bt_2cAMP produced significant increases in protein phosphorylation compared to mock infected cultures. In contrast, the extent of protein phosphorylation produced by pHSVpUC was similar to mock infected cultures.

To determine if pHSVcyr and bt_2cAMP directed the phosphorylation of similar proteins, the protein extracts were displayed on a SDS polyacrylamide gel. The resulting autoradiogram demonstrates that pHSVcyr, but not pHSVpUC, produced an increase in protein phosphorylation similar to that obtained by incubating PC12 cells with bt_2cAMP. A HSV-1 vector expressing the catalytic domain of the Ca^{++}/calmodulin dependent protein kinase II also produced an increase in protein phosphorylation, but the pattern of bands observed on SDS-polyacrylamide gels was clearly different from that obtained with pHSVcyr and bt_2cAMP. Cells infected with pHSVcyr

were incubated for one day prior to analysis, whereas the cells treated with bt_2cAMP were incubated for only 30 minutes. Thus, it is not suprising that the patterns of protein phosphorylation produced by a long term and a transient increase in cAMP are similar but not identical. Thus, the increase in cAMP levels directed by pHSVcyr is sufficient to increase protein phosphorylation in PC12 cells.

Example 8

pHSVcyr Causes an Increase in Monoamine Neurotransmitter Release from PC12 Cells

Pharmacological agents that stimulate adenylate cyclase activity are known to transiently increase neurotransmitter release from PC12 cells; therefore, the ability of pHSVcyr to direct an analogous increase in monoamine neurotransmitter release (Dopamine) from PC12 cells was investigated. 4 X 10^5 PC12 cells were infected with pHSVcyr, pHSVpUC, or mock infected, and one day later, the cells were washed once in release buffer, and then incubated for 15 minutes in 200 μl release buffer containing physiological concentrations of ions (135 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2 mM $NaPO_4$ pH 7.4, and 10 μM glucose). The amount of monamines released into the buffer was quantitated using high pressure liquid chromatography (HPLC) as described above.

The results of this experiment are summarized in Table 2. Each condition in the table was repeated at least three times, and a 10% variation was observed among samples. Dopa pHSVcyr caused an 2.85-fold increase per infected cell in dopamine release from PC12 cells relative to mock infected cell. 94% of the increase was dependent on calcium, and the increase did not require physiological activity (action potentials), as it was not affected by the sodium channel inhibitor, tetrodotoxin. This was expected for undifferentiated PC12 cells which do not extend processes or express detectable levels of sodium channels. 56 mM K+ (which depolarizes cells) and bt_2cAMP also increased neurotransmitter release, while pHSVpUC had no effect.

Thus, one day after pHSVcyr infection of undifferentiated PC12 cells, the cAMP concentration was shown to be elevated, and both protein phosphorylation and monoamine neurotransmitter release were increased. Since undifferentiated PC12 cells do not extend processes, the increase in cAMP levels caused by pHSVcyr occurred throughout the cell. It is possible that the increase in cAMP could act directly affect the neurotransmitter release machinery, to alter the amount of release, consistent with the observation that the increase in neurotransmitter release required calcium, which is required for fusion of synaptic vesicles to the plasma membrane. However, the increase in neurotransmitter release did not require physiological activity (action potentials).

TABLE 2

Dopamine Release from PC12 Cells One Day After pHSVcyr Infection

Treatment		Dopamine Released*
pHSVcyr 7.5 μl		178
pHSVcyr 15 μl		235
High K+	§	1200
2 mM dibutyryl cAMP	x	150
pHSVcyr + TT	v	200
pHSVcyr - Ca^{2+}	y	133
pHSVpUC		118
Mock		130

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* pg Dopamine Released/10^6 cells/min.

§ Typical release buffer, except 56 mM KCl and 80 mM NaCl.

x 2 mM dibutyrl cAMP added to release buffer.

y Release buffer as above, except 0.1 mM EGTA and no $CaCl_2$.

v 7.5 μl pHSVcyr; 1 μM Tetrodotoxin added to release buffer.

pHSVpUC does not contain a gene.

investigated. Cultures were infected with pHSVcyr virus, and one week later neurons containing increased cAMP-IR were observed. The increase in cAMP-IR remained localized to the cell body. Cultures infected with pHSVpUC virus, and incubated for either one day or one week, contained levels of cAMP-IR similar to those of mock infected cultures. Cultures infected with pHSVcyr virus and subjected to immunohistochemistry, using either preimmune rabbit serum or the rabbit anti-cAMP antibody preabsorbed with cAMP, contained Nf-IR cells but no cAMP-IR cells. Of note, parallel cultures infected with the same amount of pHSVcyr virus (moi. 0.1) produced similar numbers of cyr-IR and cAMP-IR neurons (approximately 10% of each), suggesting that the neurons expressing the cyr protein produced the increased cAMP levels. The increase in cAMP produced by pHSVcyr was quantitated by two methods, radioimmunoassay and incubation of cells with ^{3}H-adenine, but the both assays showed low sensitivity.

The data indicate that pHSVcyr directed an increase in cAMP levels localized to the cell bodies of neurons.

Example 10

pHSVcyr Causes an Increase in Protein Phosphorylation in Neurons

The ability of pHSVcyr to increase protein phosphorylation in neurons, presumably by activating the A kinase, was investigated. Cultures were

infected with pHSVcyr virus, pHSVpUC virus, or mock infected; one day or one week after infection, the cells were incubated for 30 minutes with $^{32}P\ PO_4$; and protein extracts were prepared. The amount of protein kinase activity was measured by determining the amount of $^{32}P\ PO_4$ incorporated into protein by TCA precipitation. Cultures infected with pHSVcyr and incubated for an additional one day or one week produced about an increase in protein phosphorylation compared to mock infected cultures, and forskolin treatment also led to increased protein phosphorylation compared to mock infected cultures. In contrast, cultures infected with pHSVpUC, and incubated for an additional one day or one week, had levels of protein phosphorylation similar to mock infected cultures.

The pattern of protein phosphorylation was visualized by electrophoresis on SDS polyacrylamide gels. pHSVcyr and forskolin activated the phosphorylation of a very similar set of proteins, and the pattern of protein phosphorylation was similar one day or one week after pHSVcyr infection. In contrast, pHSVpUC did not change the pattern of protein phosphorylation. Thus, pHSVcyr causes a stable increase in protein kinase activity in sympathetic neurons.

Example 11

pHSVcyr Stably Increases Monoamine Neurotransmitter Release from Sympathetic Neurons, and the Increase Requires Physiological Activity and Calcium

Since transient increases in cAMP produced by forskolin or bt_2cAMP increase neurotransmitter release from neurons, the effect of pHSVcyr on neurotransmitter release was investigated. The results of these experiments are recorded in Table 3. (The glia present in the cultures do not synthesize, accumulate from the medium, or secrete, monoamines.) One day or one week after infection of cultured neurons with pHSVcyr, the media was removed and the cells were incubated in a defined release buffer containing physiological ion concentrations for 15 minutes. The amount of monamines released into the buffer was quantitated using HPLC. Norepiniphrine, the neurotransmitter used by adult sympathetic neurons, was measured in initial release experiments. However, subsequent experiments measured dopamine, because it is easier to detect in this assay, and because it is a significant neurotransmitter in cultured sympathetic neurons from newborn rats. Similar results were obtained with both neurotransmitters compared to mock infected controls; the increase required calcium and physiological activity (action potentials), since it was completely inhibited by tetrodotoxin, which blocks the voltage gated sodium channel. The increase in neurotransmitter release was stable for one week, and at one week was still dependent on calcium and physiological activity.

Furthermore, the magnitude of the increase in neurotransmitter release was similar at one day and at one week. In contrast, cultures infected with pHSVpUC and incubated for an additional one day or one week showed no increase in neurotransmitter release. Forskolin; 56 mM K^+, which depolarizes neurons; and veratadine, which depolarizes neurons by opening the voltage gated sodium channel, all increased neurotransmitter release from mock infected cultures. (Depolarization substitutes for physiological activity in causing neurotransmitter release). Noteably, agents which depolarize neurons (56 mM K+ and veratadine), caused similar amounts of neurotransmitter release from pHSVcyr, pHSVpUC, and mock infected cultures. Thus, pHSVcyr causes an increase in neurotransmitter release from cultured sympathetic neurons; the increase in neurotransmitter release requires calcium and physiological activity, and is stable for at least one week.

Cultured sympathetic neurons prepared from newborn rats can be induced to change from the monoaminergic neurotransmitter system to the cholinergic system (Yamamori, T. et al., Science 246: 1412-1416, 1989). Peptide neurotransmitter production can also be altered by external stimuli (Nawa, H. and Sah, D.W.Y., Neuron 4: 279-287, 1990). Therefore, the influence of pHSVcyr on neurotransmitter type was investigated. Five days after pHSVcyr infection, greater than 99% of the cells with neuronal morphology and cyr-IR also

contained tyrosine hydroxylase immunoreactivity (TH-IR); less than 1% of the cells with cyr-IR contained choline acteyltransferase immunoreactivity (CAT-IR). In pHSVpUC or mock infected cultures, greater than 99% of the cells with neuronal morphology contained TH-IR and less that 1% of the cells contained CAT-IR. Therefore, pHSVcyr did not noticeably alter the classical neurotransmitter type of cultured sympathetic neurons.

In contrast to the results with PC12 cells, in neurons, both the cyr protein and the increase in cAMP were localized to the cell body, which is a considerable distance away from the site of neurotransmitter release at the axon terminal. Consequently, physiological activity was required to transmit a signal from the cell body to the axon terminal, and calcium was required for fusion of synaptic vesicles to the plasma membrane in the axon terminal. This suggests that pHSVcyr increased the frequency of action potentials, which originate in the cell body, thereby increasing the amount of neurotransmitter released. Consistent with this mechanism is the observation that depolarizing agents, which substitute for action potentials in affecting neurotransmitter release, cause the same amount of neurotransmitter release from pHSVcyr, pHSVpUC, and mock infected cells. If, on the other hand, pHSVcyr directly affected the neurotransmitter release machinery, then differences in the amount of neurotransmitter release between pHSVcyr and mock

infected cells might still be observed in the presence of depolarizing agents.

TABLE 3

Dopamine Release from Cultured Rat Sympathetic Neurons
One Day or One Week After pHSVcyr Infection*

Treatment	NE	DA 1 day	DA 1 wk	High K	Verat
pHSVcyr	0.25	26	30	53	35
pHSVcyr+TTx^{v}		<10	14		
pHSVcyr-Ca^{2+}y		<10	18		
pHSVpUC	0.10	13	17	51	35
Mock	0.08	11	11	49	32
Forskolin^{x}		25			
High K^{+}§	0.29				

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* pg Dopamine Released/10^6 cells/min. Dissociated neuronal cultures were prepared from superior cervical ganglia of 4 day old rats. Two weeks after plating cultures (approximately 5 x 10^5 cells) were infected with the indicated virus. One day or one week later, the medium was removed, cells were washed once in release buffer, and then incubated in 200 µl release buffer for 15 minutes. Dopamine levels were measured by HPLC. Release buffer: 135 mM NaCl, 3mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2 mM $NaPO_4$ pH7.4, and 10 µM Glucose

x 1 mM Forskolin added to release buffer.

§ Release buffer as above, except 56 mM KCl and 80 mM NaCl.

v 1 µM Tetrodotoxin added to release buffer.

y Release buffer as above, except 0.1 mM EGTA and no $CaCl_2$.

Example 12
A Protocol for Packaging pHSVlac DNA into HSV-1 Particles using a HSV-1 Deletion Mutant as Helper Virus

The Deletion Virus and Complementing Strain

HSV-1 strain 17 D30EBA virus (Paterson, T. and Everett, R.D., J. Gen. Virol., 71: 1775-1783 (1990)) and the complementing M64A cells containing the IE3 gene (Davidson, I. and Stow, E.C., J. Gen. Virol., 67: 2571-2585 (1986); Paterson, T. and Everett, R.D., J. Gen. Virol., 71: 1775-1783 (1990)), were kindly provided by Dr. Everett (University of Glasgow, Glasgow, Scotland). Figure 2 shows the extent of the deletion in D30EBA, which removes codons 83 to 1236 of the 1298 codons of the IE3 gene (McGeoch, D.J. et al., Nucleic Acids Res. 14: 1727-1745 (1986)). Figure 2 also shows the region of HSV-1 DNA containing the IE3 gene that is present in M64A cells. M64A cells contain the HSV-1 strain 17 IE3 gene and the a sequence, from nucleotide 844 in the short repeat region (McGeoch, D.J. et al., Nucleic Acids Res. 14: 1727-1745 (1986)) to nucleotide 123,018 in the long repeat region (Perry, L.J. and McGeoch, D.J. J. Gen. Virol. 69: 2831-2846 (1988)). M64A cells were constructed by transfection of BHK tk$^-$ cells with the plasmid p65, which contains the IE3 gene and the HSV-1 tk gene, and subsequent isolation by HAT selection as described (Paterson, T. and Everett, R.D., J. Gen. Virol., 71: 1775-1783 (1990); Davidson, I. and Stow, E.C., J. Gen. Virol., 67: 2571-2585 (1986))). M64A cells were grown in Dulbecco's modified minimum

essential medium with 10% fetal bovine serum; M64A cells were maintained in HAT medium until just before use.

Packaging pHSVlac DNA into HSV-1 Particles Using D30EBA Virus and Strain M64A

The HSV-1 vector, pHSVlac (Figure 1; Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988)), was packaged into HSV-1 particles using a deletion virus and complementing cell line. The D30EBA virus contains a deletion in the IE3 gene, and the M64A complementing cell line contains the HSV-1 1E3 gene. The scheme for packaging pHSVlac into HSV-1 particles, using the D30EBA virus and the M64A cell line, was adapted from the method of Geller (Geller, A.I., Nucleic Acids Res. 16: 5690 (1988)) and is outlined in Figure 3.

1.5×10^5 M64A cells were seeded on a 60 mm plate. The following day, the M64A cells were transfected (Graham, F.L. and Van der Eb, A.J., Virology 52:456-467 (1973)) with a 0.5 ml calcium phosphate co-precipitate containing 1 μg pHSVlac DNA and 9 μg salmon sperm DNA. Four hours later, the cells were treated with 15% glycerol (Parker, B.A. and Stark, G.R., J. Virol. 31: 360-369 (1979)). Following a 24 hour incubation at 37° C, the cells in each plate were infected with 8×10^6 pfu of D30EBA virus in 100 μl of medium. After 1 hour at room temperature, an additional 5 ml of medium was added to each plate, and 3 days later virus was harvested. Virus was subsequently passaged at a 1:2 dilution on M64A cells. Virus was prepared, passaged,

and titered as described (Miller, R.H. and Hyman, R.W., Virology 87: 34-41 (1978)). pHSV1lac virus was titered by determining the number of β-galactosidase positive PC12 cells (Davidson, I. and Stow, N.D., Virology 141:77-88 (1985)). D30EBA virus was titered on M64A cells and revertants to wild type were detected on CV1 monkey fibroblasts. PC12 cells were grown in RPMI 1640 containing 10% horse serum and 5% fetal bovine serum (Greene, L.A. and Tischler, A.S., Proc. Natl. Acad. Sci. USA 73: 2424-2428 (1976)) and CV1 cells were grown in Dulbecco's modified minimum essential medium with 10% fetal bovine serum.

The IE3 gene in M64A cells complements the deletion in the IE3 gene in D30EBA virus, resulting in a productive HSV-1 infection. The progeny virus from this experiment included both D30EBA virus and pHSV1lac virus, since pHSV1lac contains the sequences required for packaging into HSV-1 particles (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988)). Further, pHSV1lac is maintained in an HSV-1 virus stock due to its growth advantage over the helper virus and no genetic selection is required; pHSV1lac contains 1 HSV-1 ori in 8.1 kb, while HSV-1 contains 3 ori in 150 kb, or 1 ori in 50 kb (Spear, P.G. and Roizman, B. In: DNA Tumor Viruses, Tooze, J., Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 615-746 (1981)). Consequently, during serial passage of a virus stock, pHSV1lac becomes a larger fraction of the virus particles. To increase the titer of pHSV1lac, the virus stock from the initial packaging was passaged three additional times on M64A cells. The titers of

pHSVlac, in each of four virus stocks, increased an average of 34 fold during the first passage, and two to three fold in the two subsequent passages (Table 4). We have observed that extended serial passage of pHSVlac virus results in the production of naturally occurring defective interfering particles; therefore, these virus stocks were not passaged further.

A detailed analysis of the third passage of these four pHSVlac virus stocks was performed. Note that the growth of pHSVlac and D30EBA was substantially better than the growth of pHSVlac and ts K. The titer of pHSVlac grown with tsK was $8x10^5$ infectious particles of pHSVlac/ml (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988); Geller, A.I. and Freese, A., Proc. Natl. Acad. Sci. USA 87: 1149-1153 (1990)), while pHSVlac grown with D30EBA gave an average titer of $2x10^7$ infectious particles of pHSVlac/ml (Table 4).

The titer and reversion frequency of the D30EBA virus was determined (Table 5). D30EBA virus grew efficiently in the presence of pHSVlac DNA, and the reversion frequency of D30EBA was about $5x10^{-5}$, comparable to D30EBA virus alone. By comparison, the ts K virus has an apparent reversion frequency of about $2x10^{-3}$ at 37° C; the true restrictive temperature of ts K is 39° C (Davison, M.J. et al., J. Gen. Virol. 65: 859-863 (1984)), so the apparent reversion frequency includes virus produced due to imcomplete penetrance of the ts K allele at 37 °C.

The amount of pHSVlac virus was compared to the amount of D30EBA virus in the virus stocks, pHSVlac represented one half to two thirds of the virus stock.

Previous analyses have shown that the ratio of pHSV1ac to ts K was 0.8 (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988); Geller, A.I. and Freese, A., Proc. Natl. Acad. Sci. USA 87: 1149-1153 (1990)). Thus, the ratio of pHSV1ac to helper virus in the virus progeny is similar for ts K and D30EBA.

In summary, pHSV1ac is more efficiently packaged into HSV-1 particles using the deletion mutant D30EBA as helper virus as compared to packaging with the ts K helper virus. In addition, the reversion frequency of the deletion virus is 40-fold lower than that of ts K.

TABLE 4

pHSVlac Virus Growth Using the Deletion Mutant Packaging System

	pHSVlac titer			
passage*	pHSVlac-1	pHSVlac-2	pHSVlac-3	pHSVlac-4
transfection	$1X10^5$	$9X10^4$	$2X10^5$	$1X10^5$
p-1	$3X10^6$	$5X10^6$	$2X10^6$	4X106
p-2	$1X10^7$	$6X10^6$	$5X10^6$	$8X10^6$
p-3	$2X10^7$	$2X10^7$	$1X10^7$	$2X10^7$

*Transfection refers to the virus stock from the transfection/superinfection used to initiate packaging pHSVlac DNA into HSV-1 particles. Four transfections were performed, pHSVlac-1 through pHSVlac-4. p-1, p-2, and p-3 are the subsequent serial passages of each virus stock.

TABLE 5

The Efficiency of Packaging pHSVlac DNA into HSV-1 Particles Using the Deletion Mutant Packaging System

Virus*	HSV-1 titer M64A	HSV-1 titer CV1	Reversion Frequency	titer pHSVlac	§pHSVlac helper
pHSVlac-1	$9X10^6$	$2X10^2$	$2X10^{-5}$	$2X10^7$	2.2
pHSVlac-2	$2X10^7$	$7X10^2$	$4X10^{-5}$	$2X10^7$	1.0
pHSVlac-3	$1X10^7$	$9X10^2$	$9X10^{-5}$	$1X10^7$	1.0
pHSVlac-4	$1X10^7$	$7X10^2$	$7X10^{-5}$	$2X10^7$	2.0
D30EBA	$8X10^6$	$1X10^2$	$1X10^{-5}$	-----	---

* All pHSVlac virus stocks are from the third passage, the number after pHSVlac designates which virus stock was used. D30EBA is D30EBA virus grown alone.

The reversion frequency is the titer of D30EBA virus on CV1 cells divided by the titer of D30EBA virus on M64A cells.

§ The titer of pHSVlac virus was divided by the titer of D30EBA virus on M64 cells to give the ratio of pHSVlac virus to D30EBA virus.

Analysis of pHSV1ac DNA and D30EBA DNA in pHSV1ac virus stocks

The structures of pHSV1ac DNA and D30EBA DNA in packaged HSV-1 particles from the M64A cells were determined by Southern analysis (Southern, E.M., J. Mol. Biol. 98: 503-517 (1975)). This procedure has been described in greater detail elsewhere (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988); Geller, A.I. and Freese A., Proc. Natl. Acad. Sci. USA 87: 1149-1153 (1990)). Total cellular DNA was prepared as described by Wigler et al. (Wigler, M. et al., Cell 16: 777-785 (1979)). To detect pHSV1ac DNA, 5 μg of DNA or 10 ng of pHSV1ac DNA (isolated from E.coli HB101 as standard), were digested with 12.5 units of Eco RI overnight and resolved on 0.7% agarose gels. Following transfer to Nytran membrane (Schleicher and Schuell, Keene, NH), hybridization was performed as described (Southern, E.M., J. Mol. Biol. 98: 503-517 (1975)). The probes were radiolabeled with ^{32}P as described (Feinberg, A.P. and Vogelstein, B. Analytical Biochem. 132: 6-13 (1983)). D30EBA DNA was detected by the same procedure except DNA was digested with Eco RI and Xho I (New England Biolabs, Beverly, MA).

To detect pHSV1ac DNA, the probe was the 5.9 kb Eco RI fragment from the plasmid pCH110 (Hall, C.V. et al., J. Molec. App. Genet. 2: 101-109 (1983)). This fragment contains the pBR sequences and most of the lac Z gene, except for 133 bp at the 3' end. pHSV1ac contains three Eco RI sites, one at each end of the pBR segment and a third in the lacZ gene. 133

bp from the 3' end of the fragment (Figure 1). The 1.5 kb fragment of pHSV1ac, which contains the 3' end of lacZ gene, the SV40 early region polyadenylation site, and the HSV-1 a sequence, is not homologous to the probe, and was not detected on blots. The 4.3 kb Eco_RI fragment, which contains most of the transcription unit in pHSV1ac, and the 2.3 kb Eco_RI fragment, which contains the pBR sequences, were detected in DNA from cells infected with pHSV1ac virus. In contrast, the diagnostic bands were absent from cells infected D30EBA alone, ts K alone, or uninfected cells. Thus, pHSV1ac DNA was properly and efficiently packaged into HSV-1 particles using the deletion mutant packaging system.

The structure of the helper virus, D30EBA, was examined. D30EBA DNA was detected by the same procedure, except that DNA was digested with Eco_RI and Xho_I, and the probe was a 659 bp fragment from the HSV-1 IE3 gene (nucleotides 1065 to 1724; McGeoch, D.J. et al., Nucleic Acids Res. 14: 1727-1745 (1986)). The same DNA samples used to detect pHSV1ac DNA were digested with Eco_RI and Xho_I and subjected to Southern analysis, using a probe from the HSV-1 IE3 gene (Figure 2). This probe hybridizes to both copies of the IE3 gene in HSV-1.

Southern analysis with M64A cells infected with ts K virus, which has no deletion, was performed as a control. ts K-infected cells yielded the expected fragments of 8.5 kb and 5.5 kb. The former corresponds to the Eco_RI-Xho_I fragment spanning the junction between the short and long regions of HSV-1,

and the latter corresponds to the Eco RI fragment at the small (rightward) terminus of HSV-1 (see map in Figure 2). A band was observed above the 8.5 kb band, at 8.9 kb, and was probably due to duplication of the 401 bp a region. (The a region can be repeated at the L terminus or at the junction between the long and short regions, the a sequence is always present as a single copy at the S terminus of HSV-1 (Spear, P.G. and Roizman, B. In: DNA Tumor Viruses, Tooze, J., Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 615-746 (1981)).

Since D30EBA contains a 3462 bp deletion in the IE3 gene, the expected size of the fragments are 5.1 kb (8.5 kb in ts K) and 2.1 kb (5.5 kb in ts K). These fragments were visible in the lane from M64A cells infected with D30EBA virus and in cells infected with pHSV1ac packaged with D30EBA (containing both viruses), but were absent from uninfected CV1 cells. (A faint band just above the 5 kb bands was visible in some lanes, and was due to the complementing IE3 gene present in the M64A cells in one copy per cell). In a lytic infection, HSV-1 DNA is present at approximately 100 copies per cell (Spear, P.G. and Roizman, B. In: DNA Tumor Viruses, Tooze, J., Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 615-746 (1981)). These results indicated that the original deletion was present in the D30EBA helper virus, and that its structure was maintained.

Example 13
pHSVlac Virus Stably Expresses β-Galactosidase in Cultured Rat Sympathetic Neurons and Glia

The ability of pHSVlac virus, prepared using the deletion mutant packaging system, to stably express β-galactosidase in neurons and glia was determined. Cultured rat sympathetic neurons were infected with pHSVlac virus, and one week later an *in situ* assay for β-galactosidase was performed.

Infection of Sympathetic Neurons with pHSVlac Virus and Detection of β-galactosidase Activity

Dissociated neuronal cultures from superior cervical ganglia were prepared from four day old rats (Hawrot, E. and Patterson, P.H., Method in Enzymol. 58: 574-584 (1979)). Five days after plating, cultures were treated with 40 μM cytosine arabinoside for 24 hours to prevent glial overgrowth. One to two weeks later, cultures were infected with pHSVlac virus; at the time of infection, a culture contained approximately $5x10^5$ cells and approximately 20% of the cells were neurons.

Cultures of sympathetic neurons were infected with 2.1 μl of pHSVlac virus ($2x10^7$ infectious particles/ml), and incubated for one week. Cells were fixed with 1.0% glutaraldehyde for 15 minutes, washed three times for five minutes each with phosphate buffered saline, and reacted for β-galactosidase activity with X-Gal (Boehringer Mannheim Biochemicals, Indianapolis, IN) (Sanes, J.R. et al., EMBO J. 5 3133-3142 (1986); Price, J. et

al., Proc. Natl. Acad. Sci. USA 84: 156-160 (1987)). Cells (200-300) were scored under a phase microscope and the percentage of β-galactosidase positive cells was calculated.

X-gal positive cells which had the morphology of neurons and of glia were observed. Cultures infected with D30EBA alone, or mock infected, contained less than 0.2% X-gal positive cells. Approximately 20% of the cells in the cultures were neurons, and approximately 10% of the neurons contained β-galactosidase; since the moi was 0.1, pHSVlac virus efficiently infected neurons. These results are consistent with previous observations that pHSVlac, packaged using ts K, expressed β-galactosidase in both peripheral (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988)) and CNS neurons (Geller, A.I. and Freese, A., Proc. Natl. Acad. Sci. USA 87: 1149-1153 (1990)), as demonstrated by colocalization of β-galactosidase-like immunoreactivity and neurofilamentlike immunoreactivity.

The β-galactosidase positive cells could arise from pHSVlac persisting in one cell for a week or from horizontal transmission of pHSVlac from one cell to another. If horizontal transmission occurred, then virtually all the cells in a culture would contain pHSVlac DNA and express β-galactosidase, and both D30EBA and pHSVlac virus would be present in the culture medium. In contrast, approximately 90% of the cells were β-galactosidase negative. In addition, one week after infection of three cultures, the culture medium contained less than 10 infectious

particles of pHSVlac/ml and less than 10 pfu/ml D30EBA, below detection levels. By comparison, wild type HSV-1 kills all the cells in a culture in less than 24 hours. Furthermore, pHSVlac packaged using ts K, stably persists in cultured peripheral and CNS neurons for at least two weeks (Geller, A.I. and Breakefield, X.O., Science 241: 1667-1669 (1988); Geller, A.I. and Freese, A., Proc. Natl. Acad. Sci. USA 87: 1149-1153 (1990)). These results indicate that pHSVlac virus, prepared using the deletion mutant packaging system, efficiently infects and stably expresses β-galactosidase in cultured sympathetic neurons and glia.

Example 14

Packaging of pHSVth With the Deletion Mutant System and Expression of TH in Fibroblasts

pHSVth was packaged using the D30EBA deletion virus and the M64A complementing cell line as described in Example 12, substituting pHSVth DNA for pHSVlac DNA. Titers were similar to those of pHSVlac packaged in the deletion mutant packaging system, in which the titers were about 10X higher than packaging with ts K helper virus.

CV1 cells were infected with pHSVth and were assayed one day post-infection for TH immunoreactivity using mouse anti-human TH antibody as primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ as secondary antibody essentially as described in Example 1. However, the

multiplicity of infection was 0.1. The results were similar to those observed when the ts K virus was used to package pHSVth (Example 1). Uninfected cells and cells infected with a vector control, pHSVpUC (pHSV with the pUC19 polylinker replacing the TH insert), showed no significant staining above background. These data indicate that the pHSVth vector was packaged into virus particles by the D30EBA virus and the complementing M64A cell line. pHSVth packaged by the deletion virus system infected fibroblasts, and directed production of TH as determined by immunoreactivity. Further, the observation that the percentage of cells stained was proportional to the m.o.i. indicates that expression occurred in the majority of cells that were infected by virus.

Example 15
Neuronal Specific Expression of LacZ from the Human Neurofilament Promoter

Construction of pOHSVlac

pIEA15, which contains the ICP0 promoter of HSV-1 strain KOS, was digested with Nco I, and treated with deoxynucleotide triphosphates and Klenow fragment of DNA polymerase I to fill in the Nco I overhang. The linearized vector was then digested with Hind III, and an approximately 1 Kb fragment containing the ICP0 promoter was isolated. This fragment was ligated into the Sma I-Hind III site of bluescript, ablating the Sma I site, to make plasmid pO-Bst. pO-Bst was digested with Nco I, treated with

mung bean nuclease to ablate the Nco I site, and religated to make pO-1-Bst. The 800 bp Taq I fragment from the Sal I-Hind III fragment of p6X58, which contains the HSV-1 strain 17 origin of replication, was ligated into the Cla I site of pO-1-Bst, to make pO-2-Bst. This vector was digested with Hind III and treated with Klenow fragment to ablate the Hind III site, to make pO-3-Bst. pO-3-Bst was digested with Xba I and Sal I, releasing a 1.7 kb fragment which was purified and inserted into the Xba I and Sal I sites of pON1, to make pO-ON1. The latter was then digested with Sca I and Hind III to release a 3.5 kb Sca I-Hind III fragment. This 3.5 kb fragment was ligated with the 7 kb Sca I-Hind III portion of pHSV1ac to make pOHSV1ac.

Construction of pNFL1ac

Defective Herpes virus vector pNFL1ac was derived from vector pOHSV1ac, which carries the ICPO promoter and adjacent HSV ori S (described above). The ICPO promoter was removed by digesting pOHSV1ac with restriction enzymes Hind III and Not I and the resulting 8 kb fragment isolated. A Hind III-Not I fragment containing 2.2 kb of the human neurofilament L promoter (Julien, J.P. et al. Genes and Dev. 1: 1085 (1987)) was purified and ligated to the 8 kb fragment from pOHSV1ac, thus creating pNFL1ac. The structure of pNFL1ac is shown in Figure 4.

pNFLlac Virus Packaged in the Deletion Mutant Packaging System Directs Cell-type Specific expression of Lac Z in Neuronal Cells

CV1 monkey fibroblasts, rat PC12 cells, and primary cultures of rat superior cervical ganglia (SCG) were infected with pNFLlac virus or control virus. pNFLlac virus and control viruses, pOHSVlac and pHSVlac, were packaged in the deletion mutant virus packaging system described in Example 12. Preparation of SCG primary cultures and maintenance of CV1, PC12, and SCG cells were as described in Example 4. To detect expression of β-galactosidase (Lac Z), an in situ assay was performed. Cells were fixed with 0.5% glutaraldehyde for 15 minutes, washed three times for five minutes each with phosphated buffered saline, and reacted for β-galactosidase activity with X-Gal as described (Price, J. et al., PNAS 84: 156-160, (1987); Sanes, J.R. et al., EMBO J. 5: 3133-3142 (1986)). Cells were scored for blue color under a phase microscope.

Analysis of CV1 cells (2 X 10^5 cells/well) infected with pNFLlac virus revealed that, on average, 0.8 cells/well were positive for Lac Z. Cultures infected with pOHSVlac yielded 116 positive cells/well. Thus, the relative ratio of expression of β-galactosidase driven by the neurofilament promoter (in pNFLlac virus infected CV1 fibroblasts) as compared to that driven by the ICPO promoter (in pOHSVlac virus infected CV1 fibroblasts) was equal to 0.0069. In contrast, this relative ratio of expression in PC12 cells, which have neuronal-like

properties, is equal to 0.100, or is 14.5-fold greater than that in CV1 fibroblasts. Note that both CV1 and PC12 mock infected cells were negative for β-galactosidase. These data are summarized in Table 6.

Expression of Lac Z was also analyzed in superior cervical ganglia (SCG). About 80% of these cells were glia, and 20% were neurons. In pNFLlac virus infected SCG cells, 326 β-galactosidase positive neurons were observed and 24 β-galactosidase positive glia were observed in 14 fields (13 field diameters/well). The ratio of positive neurons to positive glia was about 14:1, thus, in the SCG cells, the human neurofilament L promoter directs cell type specific expression of β-galactosidase. In contrast, in pHSClac virus infected cells, 1183 β-galactosidase positive neurons and 4200 β-galactosidase glia were observed. Thus, the ratio of positive neurons to positive glia in pHSVlac virus infected cultures of superior cervical ganglia was about 1:4, which closely parallels the ratio of neurons to glia in the culture and suggests that the HSV-1 IE4/5 promoter in pHSVlac is expressed equally well in either cell type. Overall, in SCG cells, there was about a 56-fold increase in neuronal expression of β-galactosidase when under the control of the neurofilament promoter, compared to the IE4/5 promoter (in pHSVlac). Thus, the human neurofilament L promoter directs neuron-specific expression of the gene product of the lacZ gene from pNFLlac.

TABLE 6

β-Galactosidase-Positive Cells in CV1 Fibroblasts and PC12 Cells

	CV1 Cells (2×10^5)*	PC12 Cells (1×10^5)*
pNFL1ac	0.8/well	85/well
pOHSV1ac	116/well	840/well
pNFL1ac/ pOHSV1ac	0.0069	0.10
Mock	0	0

*Cells/well

Example 16
Fusion of the Amino-Terminal 10 Amino Acids of Human GAP-43 to β-galactosidase Targets the Protein to Neuronal Processes

The neuronal protein GAP-43 is thought to be attached to the growth cone membrane via fatty acylation of the proteins's only two cysteine residues (J. Cell. Biol. 108: 613, 1989). These cysteine residues are found within a 10-amino acid domain at the amino terminus of the molecule. To determine whether this domain alone is capable of transporting a protein to the neuronal process, and to develop a method for targeting molecules to the neuronal growth cone, a chimeric clone in which the first 10 amino acids of human GAP-43 were fused to beta-galactosidase (GAPlac) in a defective Herpes Simplex Virus (HSV-1) vector was constructed. PC12 cells, primary cultures of dissociated superior cervical ganglia, and hippocampal neurons were infected with either pHSVGAPlac virus, or pHSVlac virus, which expresses the unmodified beta-galactosidase.

Construction of pHSVGAPlac

The 5' end of the lacZ gene in pHSVlac consists of a gpt-trpS-lacZ fusion, as shown in Figure 8. The gpt portion of the fusion gene was replaced by the coding sequence of the first 10 amino acids of human GAP-43 (Kosik, K. S. et al., Neuron 1: 127-132. (1988)) as follows.

pHSVlac was digested to completion with Hind III. The Hind III cut pHSVlac was then partially digested with Asp718 and the appropriate restriction fragment was purified from a gel. This fragment was ligated with G10, which consisted of two oligonucleotides annealed with each other that contain the coding sequence for the first 10 amino acids of human GAP-43 with Hind III and Asp718 cohesive ends:

5'-AGCTTACCATGCTGTGCTGTATGAGAAGAACCAAACAG-3', and
3'-ATGGTACGACACGACATACTCTTCTTGGTTTGTCCATG-5'.

This construction resulted in fusion of the coding sequence for the first 10 amino acids of human GAP-43 in frame with the trpS-lacZ fusion. The resulting construct is shown in Figure 5.

Expression of Lac Z in NGF Differentiated PC12 Cells

$5X10^5$ PC 12 cells (Green and Tischler, PNAS 73: 2424-2428, 1976) were seeded in 5 ml on 60 mm plates coated with 0.2 ml of 100 ug/ml collagen. 12 hours later, nerve growth factor (NGF) was added to a final concentration of 10 ng/ml. 12 hours later, dibutryl cyclic AMP (bt_2cAMP) was added to a final concentration of 1mM. On day 3, $1X10^5$ infectious particles of pHSVlac virus was added to each culture. Packaging of virus was carried out as described (Geller, A. I., Nucleic Acids Res. 16: 5690, (1988)), using HSV-1 ts K virus as helper. Virus was added to the cultures and they were incubated for two days prior to assaying for β-galactosidase activity in

situ as described (Price et al., PNAS 84: 156-160, 1987; Sanes et al., EMBO J. 5: 3133-3142, 1986). Briefly, cells were fixed with 0.5% glutaraldehyde for 15 minutes, washed three times for five minutes each with phosphate buffered saline. Subsequently, they were reacted with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), a chromogenic substrate for beta-galactosidase, which generates a dark blue reaction product at the site of β-galactosidase activity.

PC12 cells differentiated in NFG for 3 days, and infected with pHSVlac showed blue reaction product in the soma only, while cells infected with pHSVGAPlac had strong staining for enzyme activity all the way down processes and into growth cones, indicating that the GAP-43/beta-galactosidase fusion protein was being targeted into growing neurites.

Expression of Lac Z was also monitored by immunofluorescence as described (Geller, A. I. and Breakefield, X. O., Science 241: 1667-1669, (1988); Geller, A. I. and Freese, A., PNAS 87: 1149-1153 (1990)). PC12 cells were differentiated for 6 days in NGF, and were infected with pHSVGAPlac. Virus was added to the culture medium two days before cells were fixed and incubated with antibody to beta-galactosidase. Following treatment with a rhodamine-conjugated secondary antibody, cultures were viewed under epifluorescence.

By immunofluorescence, cells infected with pHSVlac showed beta-galactosidase located in short neurites and growth cone-like structures in some

cases, but usually staining was restricted to the cell soma. Cells infected with pHSVGAPlac, however, consistently exhibited beta-galactosidase immunoreactivity in long neuritic networks and membrane extensions. Therefore the fusion protein was carried further along growing neurites than the enzyme alone.

Primary Cultures of Rat Neurons from Superior Cervical Ganglia (SCG) Display Beta-galactosidase Enzyme Activity in Long Thick Neurites After Infection With pHSVGAPlac Virus

Superior cervical ganglia were dissociated from 4 day-old rats and maintained for 14-21 days in vitro, then infected with virus (pHSVGAPlac or pHSVlac). 2 days later, cells were fixed and beta-galactosidase enzyme activity was demonstrated by X-gal staining (Geller, A. I. and Breakefield, X. O., Science 241: 1667-1669 (1988). Virus was packaged using the deletion virus packaging system described in Example 12, or with HSV-1 ts k as helper virus.

While SCG neurons infected with pHSVlac occasionally exhibited faint beta-galactosidase enzyme activity in proximal parts of long neurites, only neurons infected with pHSVGAPlac showed dense enzyme reaction product entirely filling thick neurites and extending into growth cones and membrane extensions. The results were comparable when cells were infected with pHSVGAPlac packaged by the ts k helper virus and the D30EBA deletion virus.

Dissociated primary cultures of E18 rat hippocampal neurons were prepared as described in Example 1 and were maintained for 12-21 days, then infected with virus two days before fixing and immunostaining as described (Geller, A. I. and Freese, A., PNAS 87: 1149-1153 (1990). Antibodies to both beta-galactosidase and Microtubule Associated Protein (MAP)-2 were applied concurrently; MAP-2 immunoreactivity was visualized with a fluorescein-conjugated antibody layer, and beta-galactosidase immunoreactivity was visualized with a rhodamine-conjugated second antibody.

Primary cultures of E18 rat hippocampal neurons exhibited granular beta-galactosidase immunoreactivity in MAP-2-positive and negative processes after infection with pHSVGAPlac, but only in the cell body after treatment with pHSVlac. MAP-2 is a marker of dendritic processes.

Thus, fusion of the amino-terminal 10 amino acids of GAP-43 to beta-galactosidase targets the chimeric protein to neuronal processes. Fusion of the aminoterminal 10 amino acids of GAP-43 to neuronal proteins provides a means of targeting recombinant molecules to the presynaptic membrane. This technology may be useful for enhancing the effects of recombinant presynaptic molecules expressed in neurons.

Example 17
pHSVpkcΔ, pHSVparv, and pHSVCaCK Increase Monoamine and Excitatory Neurotransmitter Release in Sympathetic and Cortical Neurons

Vector Constructions

Vectors containing the full length or catalytic domain of PKC (pHSVpkc, full length; pHSVpkcΔ, catalytic domain) were constructed. To detect expression of the gene products, the coding regions of the full length clone or the catalytic domain were fused to a ten amino acid peptide which is recognized by an antibody (Flag). To facilitate these fusions, a synthetic duplex encoding a 10 amino acid Flag peptide was introduced into vector pHSVpUC to make pHSVflag.

pHSVlac was digested with Eco RI and Hind III and the vector fragment was purified. The removal of the Eco RI-Hind III fragment results in the excision of most of the lacZ gene from the pHSV vector. A fragment encoding the pUC19 polylinker (Hind III-Eco RI) was inserted into the Eco RI and Hind III sites of the vector to make pHSVpUC. A synthetic duplex of the following sequence was assembled and phosphorylated:

```
                MetAspTyrLysAspAspAspAspLysSer
5'-AGCTTACCATGGACTACAAAGACGATGACGATAAATCGATAAGT-
      ATGGTACCTGATGTTTCTGCTACTGCTATTTAGCTATTCA

AAGTAAG-3'
TTCATTCTTAA-3'
```

The resulting duplex encodes the 10 amino acid Flag peptide (shown above) and has 5'-overhangs compatible with Hind III and Eco RI cut DNA. pHSVpUC was cut with Hind III and Eco RI, and the vector portion was isolated and ligated to the synthetic duplex shown above to make pHSVflag.

The rat protein kinase C β-II clone, PKC-II (Knopf, J.L. et al., Cell 46: 491-502 (1986)), was linearized with Sca I at nucleotide 994. Cla I 8-mer linkers (New England Biolabs) were phosphorylated and ligated to the Sca I-cut plasmid. The plasmid, with attached linker, was then cleaved with Cla I and Eco RI (EcoRI site in vector). The Cla I-Eco RI fragment encoding the catalytic domain of PKC was purified. Vector pHSVflag was cleaved with Cla I (AT'CGAT) and Eco RI. The pHSVflag vector fragment was isolated and ligated to the Cla I-Eco RI PKC fragment, encoding the PKC-II nucleotides from 994 through the end of the original PKC-II clone to give pHSVpkcΔ. The flag peptide is fused in frame via the Cla I linker to the coding region of PKC, beginning at nucleotide 994.

In addition, the full length rat protein kinase C β-II, coding sequence was inserted into an HSV-1 vector. For this construction, PKC-II (Knopf, J.L. et al., Cell 46: 491-502 (1986)) was cleaved at a Fnu DII site located at nucleotide 135. Cla I 8-mer linkers (New England Biolabs) were phosphorylated and ligated to the Fnu DII-cut plasmid. The plasmid, with attached linker, was then cleaved with Cla I and Eco RI. The Cla I-Eco RI fragment, which encodes the complete coding sequence of the PKC-II

clone, was isolated. Vector pHSVflag was cleaved with Cla I and Eco RI. The pHSVflag vector fragment was isolated and ligated to the Cla I-Eco RI PKC fragment to make pHSVpkc.

The starting material for cloning a rat parvalbumin cDNA into an HSV-1 vector was BPV-CaMPV (Rasmussen, C.D. and A.R. Means, Molec. Endocrinol. 3(3): 588-595 (1989)). BPV-CaMPV was digested to completion with Hind III, and partially digested with EcoRI. The Hind III-Eco RI fragment spanning the rat parvalbumin coding region was isolated. Vector pHSVpUC was digested with Hind III and EcoRI, the vector portion was isolated and was ligated the to the Hind III-Eco RI parvalbumin fragment to make pHSVparv.

A cDNA encoding the catalytic domain of the α-subunit of the calcium/calmodulin dependent protein kinase type II (CaM-K-α) from rat brain (Lin, C.R. et al., Proc. Natl. Acad. Sci. USA 84: 5962-5966 (1987)) was cloned into an HSV-1 vector. The HSV-1 construct encodes the amino terminal portion of CaM-K-α, from the nucleotides encoding the initiator methionine (codon 1) to the Xmn I site, which cuts within codon 292 (out of 478 amino acids). In an intermediate step, the fragment was inserted into pUC18, fusing the 3' end of the fragment to Hinc II-cleaved vector, thereby fusing the open-reading frame of the CaM-K fragment with the sequence of the polylinker. This procedure introduces an in frame Arg-Leu-Stop sequence to produce an open reading frame with the following structure: (CaM-K-α Met_1-Lys_{291})-(Arg-Leu)-Stop (numbering as in Lin, C.R. et al., Proc. Natl. Acad.

Sci. USA 84: 5962-5966 (1987)). A fragment encoding this trucated version of the gene was inserted downstream of the IE 4/5 promoter, replacing the lacZ sequence. The resulting construct is named pHSVCaCK.

In each case, vectors with the correct orientation were identified by restriction analysis and the structure of the constructs were confirmed by sequencing. pHSVpkc, pHSVpckΔ, pHSVparv, and pHSVpUC constructs were packaged into HSV-1 particles using the HSV-1 strain 17 D30EBA deletion virus and M64A helper cell line as described in Example 12. pHSVCaCK was packaged using a temperature sensitive helper virus (ts K).

Glutamate and Aspartate Analysis

Amino acids were analyzed using a BAS 200A binary gradient high pressure liquid chromatography (HPLC) system (Bioanalytical Systems Inc., West Lafayette, IN) with a CMA200 autoinjector (Carnegie Medicin, Stockholm, Sweden) (Shea, P.A. and W.A. Jacobs, Curr. Sep. 9: 53-55 (1989)). The amino acids were derivatized using the autoinjector prior to injection. The derivatizing reagent consisted on 100 mg O-phthalaldehyde (OPA) in 2.5 ml methanol/ 2.5 ml pH 9.6 borate buffer with 22.5 μl of tert-butylthiol. A sample to reagent ration of 8:1 v/v was used. After a 60 second reaction, the samples were injected onto the BAS 200 HPLC using a BAS Phase II 100 x 3.2 mm 3 micron C^{18} column. The mobile phases used to achieve separation were 0.1 M

acetic acid [pH 5.9], with an increasing acetonitrile/tetrahydrofuran gradient of acetonitrile from 12% to 30% and tetrahydrofuran from 1.2 to 15 %. Typically, chromatograms were complete within 15 minutes with separation of the major transmitter amino acids including aspartate, glutamate, taurine and GABA, with resolution in the majority of samples of serine, glycine, alanine, asparagine, threonine, histidine, methionine, and valine also. Combination dual electrochemical 600 mV vs. a Ag/AgCl reference electrode (detector 1) and 700 mV (detector 2) and for selected samples ultraviolet (330 nm) detectors in series were used, with peak heights recorded on a chart recorder and compared to standards. Assay sensitivity with a signal to noise ration of 5:1 ranged from 25 to 75 femtomoles.

Neurotranmsitter Release from Cultured Sympathetic and Occipital Cortex Neurons

Defective HSV-1 vectors encoding the full-length rat protein kinase C β-II (pHSVpkc), the catalytic domain of rat protein kinase C β-II (pHSVpkcΔ), rat parvalbumin (pHSVparv), and the catalytic domain of the rat α calcium/calmodulin protein kinase II (pHSVCaCK) were constructed as described above. The effect of these additional "signal transduction factors" on neurotransmitter release was investigated.

pHSVpkc and pHSVpckΔ were properly packaged into virus particles as determined by Southern

analysis. To detect expression of the protein kinase C (PKC) constructs, the coding regions were fused via a linker to the C-terminal end of a ten amino acid peptide (Flag), which is recognized by an anti-Flag antibody (e.g., M2, M5). Using the M5 anti-flag antibody, expression of both the full length and catalytic domains from pHSVpkc and pHSVpckΔ, respectively, in both cultured sympathetic and cortical neurons was confirmed. The pkcΔ protein produced by pHSVpkcΔ was expressed for at least 1 week in cultured sympathetic and occiptal cortex neurons, and was predominantly localized to cell bodies.

Release of the neurotransmitter dopamine from sympathetic neurons infected with pHSVpkc, pHSVpkcΔ, pHSVparv and pHSVCaCK was assayed as described in Example 4. Aspartate and glutamate were assayed as described above in this example. As shown in Table 7, expression of the catalytic domain of PKC from pHSVpkcΔ led to an increase in release of both monoamine (dopamine) and excitatory amino acid (aspartate, Asp; glutamate, Glu) neurotransmitters in the presence of depolarizing agents such as high potassium (K) (Table 7) and veratadine (not shown), which mimic a state of high neuronal activity. This increase in neurotransmitter release required calcium. Cultures infected with pHSVpkc, encoding the full length protein kinase C, a pHSVpUC control, or mock infected cultures (mock) did not show a similar increase in neurotransmitter release in the presence of depolarizing agents or in the basal

state. In the basal state, the catalytic domain of PKC expressed by pHSVpkcΔ had no detectable effect on neurotransmitter release under these conditions.

The data in Table 7 show the effects of PKC on neurotransmitter release one day after infection. Similar data were obtained one week after infection, indicating that infection with pHSVpckΔ can cause long term increases in neurotransmitter release. The ability of pHSVpkcΔ to affect neurotransmitter release in both sympathetic and cortical neurons suggests that PKC can alter some general, and therefore conserved, aspect of neuronal function which is likely to operate in most neurons. Possibly, the full-length protein kinase C had no observable effect on the cells in this assay because the wild type portien kinase C is strictly regulated.

Sympathetic and cortical neurons infected with the catalytic domain of the calcium/calmodulin dependent protein kinase II displayed a similar pattern of neurotransmitter release (i.e., increased neurotransmitter release in the presence of depolarizing agents, but no detectable increase in the basal state), but to a lesser extent.

TABLE 7

NEUROTRANSMITTER RELEASE FROM CULTURED SYMPATHETIC AND OCCIPITAL CORTEX NEURONS 1 DAY AFTER pHSV VIRUS INFECTION

Condition	Sympathetic Neurons*		Cortical Neurons#			
			low K		high K	
	low K	high K	Asp	Glu	Asp	Glu
pHSVpkc	<8	13	63	76	127	115
pHSVpkcΔ	<8	130	93	73	351	253
pHSVpUC	<8	13	76	72	144	127
mock	<8	20	87	81	149	106

* pg Dopamine released/10^6 cells/min.

\# nM Asp or Glu.

Cultures of sympathetic neurons prepared from newborn rats were maintained for at least two weeks before infection with virus or mock infection. One day after infection, the medium was removed and the cells were incubated fpr 15 minutes at 37 °C in release buffer containing physiological ion concentrations. The release buffer was removed and assayed for neurotransmitters.

The effects of a calcium binding protein on neuronal function were also examined. Parvalbumin is a calcium binding protein primarily localized to rapid firing GABAergic neurons. Therefore, a vector expressing parvalbumin, pHSVparv, was constructed and used to introduce parvalbumin into neurons which do not normally contain it. Expression of parvalbumin in cultured sympathetic and cortical neurons was detected using an antibody directed against parvalbumin. Table 8 shows the effect of parvalbumin on neurotransmitter release in these cell types. As shown in Table 8, parvalbumin directed a long term increase in neurotransmitter release from both sympathetic and cortical neurons in both the basal state and following depolarization.

These studies indicate that stable activation of different signal transduction pathways has specific and distinct long term effects on neuronal function. Activation of the cAMP pathway by infection with pHSVcyr virus increases neurotransmitter release in the basal state, but not following depolarization (Example 11). In contrast, activation of either calcium dependent protein kinase pathway by infection with pHSVpkcΔ (Table 7) or pHSVCaCK increases release following depolarization, but not in the basal state. Additionally, expression of the calcium binding protein parvalbumin increases release in both the basal state and following depolarization (Table 8). These studies indicate that stable activation of signal transduction pathways can cause specific long term effects on the function of normal neurons.

TABLE 8

NEUROTRANSMITTER RELEASE FROM CULTURED SYMPATHETIC AND OCCIPITAL CORTEX NEURONS 1 DAY AFTER pHSVparv INFECTION

	Sympathetic Neurons*		Cortical Neurons#			
			low K		high K	
Condition	low K	high K	Asp	Glu	Asp	Glu
pHSVparv	22	30	100	160	290	534
pHSVpUC	<8	13	76	72	144	127
mock	<8	18	87	81	149	106

*pg Dopamine released/10^6 cells/min.
#nM Asp or Glu.

The experiment was performed as in Table 7.

Example 18
Rotational Behavior of Rats Stereotactically Injected with pHSVpkcΔ Virus

The Rat Rotation Model

Ungerstedt and Arbuthnott originally described asymmetric rotation of rats following the unilateral lesioning of the substantia nigra (Ungerstedt, U. and G.W. Arbuthnott, Brain Res. 24: 485-493 (1970)). Ungerstedt later reported that the partial unilateral denervation of the ascending mesostriatal dopamine system produced an upregulation of post-synaptic dopamine (DA) receptors on the lesioned side which developed over a 7 to 14 day period (Ungerstedt, U., Acta. Physiol. (suppl.) 367: 69-93 (1971)). This upregulation was a presumed homeostatic compensation for the reduced afferent dopaminergic input. The asymmetry in dopamine receptors did not influence spontaneous behaviour. However, rotational behavior was induced following administration of a drug which acts directly on post-synaptic DA receptors (e.g., the dopamine agonist apomorphine), or alternatively, of a drug which acts indirectly by increasing synaptic levels of endogenous dopamine (L-dopa or amphetamine). Apmorphine-treated animals rotate away from the lesioned side, because the increased post-synaptic activity ipsilateral to the lesion produces contralateral turning. In contrast, amphetamine produces ipsilateral turning, as it produces a much

greater increase in extracellular dopamine in the intact striatum as compared with the lesioned side.

Although primarily used as a behavioural marker of a lesioned substantial nigra, the rotation produced by apomorphine will reveal asymmetry in striatal post-synaptic receptors caused by other mechanisms, with rotation occurring in the direction of the striatum with the reduced number of post-synaptic DA receptors (as compared to the contralateral side). In addition to exhibiting plasticity by increasing receptors in response to lesioning, the striatal dopamine system is able to downregulate these receptors in response to increased dopaminergic transmission.

The following experiment was designed to test whether stereotactic injection of pHSV constructs encoding all or part of an active signal transduction factor, such as the catalytic domain of protein kinase C, into the substantia nigra can cause an increase neuronal activity and stimulated dopamine release sufficient to result in downregulation of post-synaptic dopamine receptors in the striatal follower cells. If so, injection of pHSVpkcΔ into the substantia nigra of an animal would induce the animal to rotate toward the injection side upon treatment with the dopamine agonist, apomorphine.

Stereotactic Injection

Male Sprague Dawley rats weighing 250 to 300 grams were anesthetized with chloral hydrate (400

mg/kg, intraperitoneally). The rats were placed in a Kopf stereotactic frame and the skull was exposed. A hole was drilled above the right substantia nigra pars compacta, AP +3.5 from lambda, 2.15 lateral, flat skull (Paxinos, G. and C. Watson, (1986) The Rat Brain in Stereotaxic Coordinates, 2nd edition, (Academic Press: New York, NY)).

Two sites within the right substantia nigra were chosen to ensure that both medial and lateral aspects of the nucleus were reached. At the medial site, AP +3.5, L 1.9, the needle bevel was orientated rostrally and the needle was implanted 7.1 mm ventrally from the dural surface. At the lateral site, AP +3.5, L 2.3, the needle bevel was facing laterally and was implanted at a depth of 6.8 mm from the dura. At each site, 2 μl of pHSVpkcΔ, encoding the catalytic domain protein kinase C or pHSVpUC (control) was injected over 5 minutes. The needle left in place for an additional 3 minutes and then withdrawn over the course of a 5 minute period.

For these experiments, defective HSV-1 constructs pHSVpkcΔ and pHSVpUC were packaged into virus particles using the D30EBA deletion virus and M64A helper cell line. The virus was concentrated approximately 80-fold by the following procedure (all manipulations were performed at 4°C): 10 ml of virus was centrifuged at 10,000 X g for 10 min. The supernatant was layered onto 2 ml of 25% sucrose in calcium and magnesium free-PBS and centrifuged at 77,000 X g for 12-16 hours. The pellet was

resuspended in 100 μl of calcium and magnesium free-PBS to make a concentrated virus stock.

Apomorphine-Induced Rotational Behavior

At 4 to 5 day intervals postoperatively, rats were tested for rotational behavior. Rats were administered apomorphine (1 mg/kg) intraperitoneally and placed in a hemispherical plexiglass rotometer. This dose of apomorphine elicits rotational behaviour in rats with significant asymmetry of striatal dopamine receptors (Hefti, et al., Pharmacol. Biochem. Behav. 12: 185-188 (1980)).

Neither of two rats which received control (pHSVpUC) virus had any asymmetrical rotation. On day twelve, following implantation of virus, one of the two rats injected with pHSVpkcΔ rotated ipsilaterally to the injection (clockwise) a total of 15 times during the 5 minute interval from 15 to 20 minutes after apomorphine injection. This apomorphine-induced rotational behaviour was stable during 4 additional tests (15 to 20 rotations per 15 fifteen minutes). The latest rotational test, was 6 weeks following the pHSVpkcΔ stereotactic injection. All animals stereotactically injected with the defective HSV-1 vectors and deletion helper virus were healthy at this time, supporting the safety of the system for gene therapy.

The clockwise apomorphine-induced rotation is consistent with an asymmetry of receptor status, with the greater post-synaptic density contralateral to the direction of rotation (i.e., on the left).

These results are consistent with the interpretation that the pHSVpkcΔ efficiently infected significant numbers of nigral dopamine cells in the right nucleus. Furthermore, the data suggest that these cells expressed an active catalytic domain of PKC and, as a result, their firing frequency and dopamine release was increased. Post-synaptic dopamine receptors on striatal follower cells were down regulated in response, and upon the administration of apomorphine, this receptor asymmetry was reflected in apomorphine-induced rotational behavior.

Example 19

pHSVngf Virus Directs Efficient Synthesis of Biologically Active NGF in Cultured Cells

Construction of pHSVngf

The rat NGF gene was kindly provided by Dr. G. Heinrich, Boston University; exon IV encodes all of pre-pro NGF. A hybrid mouse/rat NGF mini-gene was constructed which lacks the sequences from 90 bp into the first intron to a point within intron III which is 277 bp before the start of exon IV. In addition, because the rat NGF mRNA contains an AU rich sequence in its 3' untranslated region (Scott et al., Nature 302: 538-540 (1983)) that may predispose it to rapid turnover, the 3' untranslated (3' UT) region was replaced with the 3' UT region from the human growth hormone (HGH) gene, whose mRNA is long lived. In particular, the portion of the 3'

UT region of the rat NGF gene 6 base pairs after the NGF stop codon) was replaced with nucleotides 1859-2657 of the HGH gene 3' UT region Seeburg, P., DNA 1: 239-249 (1982)). The resulting ~1.9 kb NGF mini-gene was inserted into a defective HSV-1 vector to make pHSVngf (Figure 9).

Structure of the NGF Minigene

All pieces were sent by Dr. Gerhard Henreich. The NGF minigene used in pHSVngf is composed of three parts from 5' to 3': (1) a non-coding Exon I-Intron I fragment (107 bp) from the mouse NGF gene (Selby, M.J. et al., Mol. Cell. Biol. 7: 3057-3064 (1987)); (2) the rat Intron III-Exon IV (1022 bp); and (3) a fragment (799 bp) from the human growth hormone gene containing the 3' untranslated region and 3' flanking sequences.

Construction Sequence

pGEM7z was digested with Xho I, the Xho I site was made blunt by a fill-in reaction with Klenow and dNTPs, and the product was digested with Bam HI. The mouse Exon I-Intron I fragment was excised with Hae II and Bam HI and inserted into pGEM7z at the blunted Xba I site and Bam HI site. The resulting pGEM7z construct was then cut with Xba I and Bam HI to release a 118 bp fragment carrying 107 bases of mouse NGF Exon I- Intron I.

A genomic Eco RI fragment containing the rat Exon II-Exon IV sequence (starting with the nucleotide 277 bp before exon IV) which encodes rat

NGF Intron III-Exon IV region was cleaved with Pst I (at a site 1024 bp downstream of the 5'-Eco RI site), and made blunt by removing the 3' overhang to make a 1022 bp fragment. This fragment was ligated to a human growth hormone Bgl II- Eco RI fragment (nucleotides 1859-2657), which had been made blunt at the Bgl II site. The fusion fragment was inserted into the Eco RI site of Bluescript (Stratagene) and was excised as a 1.8 kb Bam HI- Sal I fragment. This 1.8 kb Bam HI-Sal I fragment and the mouse 118 bp Exon I-Intron I Xba I-Bam HI fragment were cloned into a Bluescript vector which had been cut with Xba I and Sal I, to make the NGF minigene. The minigene was then excised from bluescript as a ~1.9 kb Xba I-Sal I fragment and cloned into the Xba I and Sal I sites of pHSVpUC.

Virus Production

pHSVngf was packaged into HSV-1 particles using the D30EBA deletion mutant and M64A helper cell line packaging system (Example 12). The titer of the pHSVngf virus stock was 5×10^5 infectious particles/ml and 1×10^6 plaque forming units of D30EBA (helper virus).

Cell Culture and Infection Protocol

NIH 3T3 cells were cultured in DMEM containing 10% fetal calf serum and 5% horse serum, at 37°C in an atmosphere of 5% CO_2. PC12 cells were cultured in 90% DMEM and 10% fetal calf serum. Cells were plated in wells (5×10^4 cells/cm^2) and allowed to

grow for 48 hours before infection. Cells were infected with 20 μl of unconcentrated virus. Eight hours later the virus containing media was removed, and 1.0 ml of fresh media was added. For determinations of the amount of NGF secreted by ELISA, the media was harvested twenty four hours later; alternatively, the media was harvested 48 hours later for determining the ability of the secreted NGF to support sympathetic neuron survival in a bioassay.

pHSVngf Virus Can Direct the Synthesis of NGF in Cultured Fibroblasts

The ability of pHSVngf virus to infect 3T3 fibroblasts and to direct the synthesis of NGF in these cells was tested. The amount of immunoreactive NGF that was synthesized in 3T3 fibroblasts was determined. 3T3 cells were infected with pHSVngf virus or mock infected. Eight hours later the medium was replaced with 1.0 ml fresh medium, and 24 hours later the medium was harvested. The amount of NGF secreted into the media was quantitated in a 2 site ELISA, using polyclonal antibodies (Dr. D. Sinicropi, Genentech, Inc.) directed against human NGF, and a murine NGF standard. The media contained ~12 ng/ml of NGF (Table 9). In 3 repetitions of the experiment the amount of NGF secreted varied from 10 - 34 ng/ml.

The 2-site ELISA was also used to determine the rate of NGF production in pHSVngf-infected 3T3 fibroblasts. Infection of 3T3 cells with 20 μl of

pHSVngf virus, containing approximately 50,000 infectious particles (determined by immunostaining

TABLE 9

pHSVngf Infected 3T3 Cells Secrete NGF into the Culture Medium as Measured by ELISA

Dilution of Medium	NGF (ng/ml)
1:4	10.16
1:8	11.24
1:16	12.16
1:32	12.8
1:64	11.84
Average of dilutions	11.64

2×10^5 3T3 cells were infected with 0.2 ml of pHSVngf virus. 8 hours later the media was removed and replaced with 1 ml of fresh medium. 24 hours later the media was harvested and NGF levels were determined by a 2 site ELISA. Samples were diluted with assay diluent and compared with a murine NGF standard.

infected 3T3 cells with NGF antisera) results in the production of 230 pg of NGF/hr/10^4 infected cells on the third day after infection. PC12 cells gave quantitatively similar results. This rate is greater than the production rate reported for the retroviral mediated stably transfected NGF producing fibroblasts (50 pg/hr per 10^5 cells in culture; Rosenberg, M.B. et al., Science 242: 1575-1578 (1988)).

Measurements of NGF by Bioassay

A neonatal sympathetic neuron survival assay (Lindsay, R.M., Nature 282: 80-82 (1979)) was used to measure the amount of biologically active NGF produced after infection of cultured cells with pHSVngf virus. NIH 3T3 cells and PC12 rat pheochromocytoma cells were cultured and infected with pHSVngf virus or mock infected as described above. The inoculum was replaced with fresh medium, and 48 hours later the media was harvested and assayed for NGF bioactivity in the neonatal sympathetic neuron survival assay.

In brief, approximately 4 x 10^4 cells from the superior cervical ganglia (SCG) of newborn rats were plated on collagen and cultured with media comprised of 50% Hams F_{12} containing 10% fetal calf serum and 50% conditioned media from the infected cells. Twenty-four hours after plating, rapidly dividing cells were killed by treatment with 5 μM cytosine arabinoside and neuron survival (= surviving neurons) was scored 72 hours later by counting at

least 10 high power fields. Conditioned media from uninfected control cultures provided a negative control. As a positive control, cultures received 100 ng/ml exogenous NGF (NGF added) with no conditioned media.

As shown in Table 10, the conditioned medium from pHSVngf-infected 3T3 cells or pHSVngf-infected PC12 cells dramatically increased sympathetic neuron survival as compared with media from mock infected cultures. The data indicate that infection of both PC12 and NIH 3T3 cells with increasing amounts of pHSVngf virus particles results in progressively greater amounts of secreted bioactive NGF, as assayed by sympathetic neuron survival.

TABLE 10

Media from pHSVngf-Infected 3T3 or PC12 Cells Can Support the Survival of Cultured Sympathetic Neurons

Cell Type	Volume Virus (μl)	NGF added (ng/ml)	# Surviving Neurons
NIH 3T3	0	0	100
	10	0	8,400
	20	0	18,200
	0	100	29,600
NIH 3T3	0	0	0
	10	0	17,100
	20	0	30,000
	0	100	32,650
PC12	0	0	0
	10	0	2,000
	20	0	13,400

The neuron counts represent the mean of duplicate plates.

Infection with pHSVngf Increases Survival of Primary Neonatal Sympathetic Neurons in Culture

Primary neonatal sympathetic neurons were infected with pHSVngf virus in culture in order to determine whether the pHSVngf construct would permit survival in the absence of exogenously added NGF. Approximately 10,000 neonatal sympathetic neurons were plated in each well in the presence of 100 ng/ml of NGF. The cultures were treated with cytosine arabinoside for 36 hours and at 48 hours the cultures were infected with either 20 μl of pHSVngf or of pHSVlac, or mock infected. The virus containing media was removed 12 hours later and every 2 days the neurons were fed with fresh media without exogenous NGF. Neuron counts were performed at days 4 and 6 days and the mean number of surviving neurons from duplicate wells was determined.

At 4 days, a mean number of about 6000 surviving neurons were present in pHSVngf virus infected wells, while in the mock infected or pHSVlac virus infected control wells, only about 2500 surviving neurons were present. At 6 days, about 5000 surviving neurons were present in pHSVngf virus infected wells compared with about 1000 surviving neurons in the mock infected or pHSVlac virus infected control wells. The numbers of neurons surviving at either 4 or 6 days in pHSVngf infected cultures were significantly greater ($P < 0.05$) than mock or pHSVlac infected cells. Positive control cultures containing 100 ng/ml NGF had mean

neuron survival numbers of 3,400 and 3,550 on days 4 and 6, respectively. These results indicate that, in the absence of exogenous NGF required for maintenance in culture, pHSVngf infection can prolong the survival of primary sympathetic neurons.

Example 20
Expression of NGF In Vivo, From a Defective HSV-1 Vector, Prevents Effects of Axotomy on Sympathetic Ganglia

Virus Production and Concentration

pHSVngf was packaged into HSV-1 particles using the D30EBA deletion mutant and M64A helper cell line packaging system (Example 12). The titer of the pHSVngf virus stock was 5×10^5 infectious particles/ml and 1×10^6 plaque forming units of D30EBA (helper virus). For these in vivo experiments, virus was concentrated approximately 80-fold by the following procedure (all manipulations were performed at 4°C): 10 ml of virus was centrifuged at 10,000 X g for 10 min. The supernatant was layered onto 2 ml of 25% sucrose in calcium and magnesium free-PBS and centrifuged at 77,000 X g for 12-16 hours. The pellet was resuspended in 100 µl of calcium and magnesium free-PBS to make a concentrated virus stock.

Direct Injection of pHSVngf Virus into SCG and Subsequent Axotomy of the SCG

Adult rats (female, Sprague Dawley) weighing 175-225 grams were anesthetized with halothane (4.5% v/v for induction, 3.5% v/v for surgery with 5 liters oxygen/minute), and the SCG was exposed unilaterally. Two μl of concentrated pHSVngf virus, pNFlac virus or saline were injected directly into the ganglion using a Hamilton syringe equipped with a 27 gauge beveled needle. Surgical wounds were closed with surgical staples. Four days after injection, axotomies were performed on the injected ganglia as previously described (Kessler, J.A. and Black, I.B., Brain Res. 171: 415-424 (1979)). Briefly, the ganglia were exposed and both major efferent branches of the SCG (internal and external carotid branches) were cut close to the ganglia, without severing visible vasculature. The wounds were again closed with surgical staples. In all animals, the contralateral, unperturbed, SCG ganglion served as an internal control. Ten days later, both the ipsilateral (experimental) and contralateral (control) ganglia were removed and assayed for TH activity.

Measurement of Tyrosine Hydroxlase Activity

Each ganglion was homogenized in 75 μl of distilled water in a glass/teflon homogenizer. Ten μl of each homogenate were assayed for TH activity by previously published methods (Kessler, J.A. and I.B. Black, Brain Res. 171: 415-424 (1979)) using tetrahydrobiopterin as cofactor.

pHSVngf Virus Can Prevent Some of the Effects of Axotomy of SCG in vivo

Injury to the nervous system frequently disrupts the normal interactions between neurons and their target cells. In cases where the target cell provides the innervating neuron with a neurotrophic factor, such disruptions lead to a deficiency in neurotrophic factor supply. NGF is synthesized and secreted by target tissues of sympathetic neurons. NGF is taken up by sympathetic neurons and retrogradely transported to the cell soma, where it functions, in the adult, to maintain the noradrenergic neurotransmitter system, in part by stimulating TH synthesis.

Previous studies have shown that after axotomy (a nervous system injury), the amount of TH within sympathetic neurons of the superior cervical ganglion (SCG) declines as a consequence of diminished NGF levels. Treatment with NGF at the time of axotomy prevents this decline in TH levels. The SCG of the adult rat contains the cell bodies of sympathetic neurons whose axons project to target tissues in the head and neck which produce NGF. Unilateral axotomy of a SCG interrupts its NGF supply and results in an ipsilateral decline in TH activity over a 10 day period. As local or systemic administration of NGF can prevent the decline in TH activity observed in the axotomized ganglion, the effect of direct injection of a SCG with pHSVngf virus was studied for a similar protective effect.

Virus was packaged and concentrated as described above. In each rat, a SCG was unilaterally infected with concentrated pHSVngf virus (2 μl). Four days later, SCG axotomy was performed ipsilateral to the injection as described above. Ten days later, TH activity was assayed in both the injected/axotomized and the contralateral, control ganglia. To control for non-specific effects due to the defective HSV-1 vector system, the same experiment was performed with 2 μl concentrated pNFlac (Example 17), another defective HSV-1 vector which expresses E. coli β-galactosidase instead of NGF, or with saline. A total of 10 rats were injected with pHSVngf virus and 9 rats were injected with pNFlac virus.

Axotomy of ganglia injected with pNFlac control virus reduced TH activity by 50% ($p = 0.02$) compared with the contralateral control ganglion (Figure 10). In contrast, injection of ganglia with pHSVngf virus prevented the decline in tyrosine hydroxylase activity after axotomy; moreover, pHSVngf injection actually led to an 18% increase in TH activity in axotomized ganglia as compared with the contralateral control. The difference observed between the pNFlac virus + axotomy group and all aother groups is statistically significant ($p = 0.02$ by ANOVA). Similar results were obtained in another experiment in which pHSVngf virus injection, but not saline injection, was able to prevent the decline in TH activity produced by axotomy.

These data demonstrate that pHSVngf virus stimulates TH activity in neurons in the axotomized SCG, thereby preventing some of the deleterious effects produced by axon injury (e.g., decline in TH activity). The data indicates that direct infection of cells in the SCG with pHSVngf virus, and subsequent expression of NGF results in the synthesis of NGF in sufficient quantities to prevent the decline in TH levels caused by axotomy.

Since a SCG contains both neurons and non-neuronal cells, direct injection of pHSVngf virus into a SCG could result in the infection of both neuronal and non-neuronal cells. Defective HSV-1 vectors can infect a large variety of different cell types including neurons and non-neuronal cell types. In addition, many cell types possess the biosynthetic machinery to synthesize and secrete bioactive NGF. Although the contribution made by each infected cell type to the total amount of NGF produced is not known, the data demonstrate the ability of pHSVngf virus to infect different cell types (e.g., fibroblasts, pheochromocytoma cells, SCG cells), to direct the production of large amounts of biologically active NGF after infection in these cell types, and to modulate enzyme levels (e.g., TH levels) in the injured nervous system.

Example 21
pHSVngf Infection Increases Choline Acetyl Transferase Activity in Cultured Striatal Neurons

In the adult CNS, a trophic role for NGF has been demonstrated for the group of ascending basal forebrain cholinergic neurons that synapse on NGF-producing hippocampal neurons. When these cholinergic neurons are disconnected from their NGF-producing target cells by axotomy, they degenerate and their content of choline acetyl-transferase (ChAT) decreases. The decrease in ChAT positive neurons can be reversed to a certain extent by the administration of exogenous NGF (Kromer, L.F., Science 235: 214-256 (1987); Gage, F.H. et al., Neurosci. 19: 241-256 (1986)) or the transplantation of fibroblasts genetically engineered to secrete NGF (Rosenberg, M.B. et al., Science 242: 1575-1578 (1988)). Because cholinergic neurons increase their content of ChAT in response to NGF, it was next determined whether pHSVngf infection of central cholinergic striatal neuron cultures would result in the expression of sufficient quantities of NGF to alter their biochemical phenotype.

Approximately 1.5 X 10^6 rat embryonic day 17 striatal neurons were plated in each of 48 wells. Twenty-four hours after plating, the cultures were mock, PHSVlac (20 μl of virus), or pHSVngf infected. Two different volumes (5 and 10 μl) of pHSVngf virus were used (See Example 19 for virus production and titer.) A separate group of control cultures were

maintained with 100 ng/ml of exogenous NGF. Each group consisted of 6-8 independent cultures. Ten days after infection, the cultures were harvested and protein concentration was determined. ChAT assays were performed as described by Fonnum (Fonnum, F. Biochem. J. 115: 465-472 (1969)).

As shown in Figure 11, the cultures infected with pHSVngf virus showed significant increases in ChAT activity relative to mock infected cultures. These results indicate that infection of cholinergic neurons with pHSVngf virus results in the production of quantities of NGF sufficient to alter their biochemical phenotype. The ChAT activity in cultures infected with 10 μl pHSVngf was significantly greater ($p < 0.05$) than mock infected or pHSV1ac infected cultures.

Example 22
Low Affinity Nerve Growth Factor Receptor Mutants

Three defective HSV-1 virus constructs were made encoding (1) the human low affinity nerve growth factor receptor (NGFR), (2) a truncated mutant NGFR which has a premature termination signal shortly after the transmembrane domain coding sequence, and (3) a mutant version of the NGFR which contains a large deletion in the ligand binding domain.

Vector Constructions

A construction encoding the full length human NGFR, $p75^{NGFR}$ (Johnson, D. et al., Cell 47: 545-554

(1986)), in an HSV-1 vector was made. A 2.3 kb Eco RI-Eco RI fragment, which retains only a small portion of the 3' untranslated region, including the polyadenylation signal, was isolated from a full-length human NGF receptor cDNA clone (E1; Johnson, D. et al., Cell 47: 545-554 (1986)). The Eco RI overhangs of the fragment were made blunt by filling in the ends in a reaction using dNTPs and the Klenow fragment of DNA polymerase. pHSVpUC (see Example 17 was digested with HindIII, and made blunt in the same manner. The Eco RI-Eco RI cDNA fragment encoding $p75^{NGFR}$ was inserted into the vector to make pDB1. Plasmids having the insert in the correct orientation (i.e., with the 5' end of the cDNA fused to the HSV IE 4/5 promoter) were identified by restriction analysis.

A construction which encodes the receptor lacking a cytoplasmic domain was constructed. Hempstead et al. have described the construction of a mutant cDNA of the NGFR having a stop codon four amino acids after the transmembrane domain at amino acid 940 (pXba; Hempstead, B.L. et al., J. Biol. Chem. 265: 9595-9598 (1990)). The Eco RI-Eco RI fragment encoding this mutant cDNA was isolated from pXba, and the ends were made blunt by filling in with the Klenow fragment of DNA polymerase and dNTPs. The fragment was inserted into pHSVpUC which had been cleaved with Hind III and made blunt to make pDB2. Plasmids with the correct orientation were identified by restriction analysis.

A third construction was designed which encodes a mutant form of the low affinity NGF receptor which

lacks the presumptive ligand binding domain. In particular, the 154 amino acids (462 base pairs) between residues 7 and 162 of the NGFR are deleted (7 and 162 are present). To generate this variant the 2.3 kb Eco RI-Eco RI fragment containing the $p75^{NGFR}$ coding sequence was cloned into the Eco RI site of pT7/T3 (Bethesda Research Laboratories). The resulting plasmid was digested with Stu I and was partially digested with Sau 3A. The Sau 3A overhang was filled in using Klenow fragment and dNTPs. The construct was then religated to delete the region between the StuI and Sau 3A sites. The recircularized plasmid was digested with Eco RI to release the NGFR cDNA fragment. The fragment was isolated, and the ends were made blunt by treatment with Klenow fragment and dNTPs. pHSVpUC was cleaved wtih Hind III, the overhangs were made also blunt with Klenow, and the Eco RI-Eco RI fragment was inserted into this vector to make pDB3. Plasmids which had sustained a 462 bp deletion were identified by restriction digestion. The structure of pDB3 was also confirmed by DNA sequencing.

CLAIMS

1. A method of altering the level of a gene product in cells, comprising the steps of:
   (a) inserting a nucleotide sequence encoding the gene product into a defective Herpes virus vector, the vector having a promoter that is able to functionally express the gene product, to make a defective Herpes virus vector construct;
   (b) introducing the construct into a cell line together with a neurotropic Herpes virus deletion mutant helper virus, said cell line being one which complements the defect of the deletion mutant helper virus, thereby a productive infection occurs and the construct is packaged into virus particles, thereby producing packaged virus construct; and
   (c) infecting cells with the packaged virus construct.

2. A method of altering the level of a gene product in cells to alter a disease state, comprising the steps of:
   (a) inserting a nucleotide sequence encoding the desired product into a defective Herpes virus vector, the vector having a promoter that is able to functionally express the gene product, to make a defecti e Herpes virus vector construct;

(b) introducing the construct into a cell line together with a neurotropic Herpes virus deletion mutant helper virus, said cell line being one which complements the defect of the deletion mutant helper virus, whereby a productive infection occurs and the construct is packaged into virus particles, thereby producing a packaged virus construct; and

(c) infecting cells with the packaged virus construct.

3. A method of altering the level of a gene product in a cell type-specific manner, comprising the steps of:

(a) inserting a nucleotide sequence encoding the desired product into a defective Herpes virus vector, having a promoter which is a cell type-specific promoter, and which is able to functionally express the gene product, to make a defective Herpes virus vector construct;

(b) introducing the construct into a cell line together with a neurotropic Herpes deletion mutant helper virus, said cell line being one which complements the defect of the deletion mutant virus, whereby a productive infection occurs and the construct is packaged into virus particles, thereby producing a packaged virus construct; and

(c) infecting cells with the packaged virus construct.

4. The method of Claim 3 wherein the cell-type-specific promoter is a neural cell-specific promoter.

5. The method of Claim 4 wherein the nucleotide sequence expressed from the neural cell-specific promoter encodes a neurotrophic factor.

6. The method of Claim 3 wherein the cell-type-specific promoter is a neuronal cell-specific promoter.

7. The method of Claim 6 wherein the neuronal cell specific-promoter is the human neurofilament L promoter.

8. The method of Claim 6 wherein the nucleotide sequence expressed from the neuronal cell-specific promoter encodes tyrosine hydroxylase.

9. The method of Claim 6 wherein the nucleotide sequence expressed from the neuronal promoter encodes a signal transduction factor or portion thereof.

10. The method of Claim 9 wherein the nucleotide sequence encodes a portion of a signal transduction factor and the factor is the nucleotide sequence comprising nucleotides 5465-6968 of adenylate cyclase from yeast.

11. The method of Claim 3 wherein the nucleotide sequence additionally includes an intracellular targeting sequence in the vector to direct the gene product to a desired location in the cell.

12. The method of Claim 11 wherein the desired location in the cell is a neuronal process.

13. The method of Claim 12 wherein the nucleotide sequence encoding an intracellular targeting sequence is the human GAP-43 targeting sequence.

14. The method of Claim 3 wherein the deletion virus is the D30EBA virus and the complementing cell line is M64A.

15. The method of Claim 3 wherein altering the level of a gene product is a cell-type-specific manner affects a disease state.

16. A method of altering the level of a gene product in a cell type-specific manner, comprising the steps of:

(a) inserting a nucleotide sequence encoding the desired product into a defective HSV-1 vector, having a promoter which is a cell type-specific promoter, and which is able to functionally express the gene product, to make a defective HSV-1 vector construct;

(b) introducing the construct into a cell line together with an HSV-1 deletion mutant helper virus, said cell line being one which complements the defect of the deletion mutant virus, whereby a productive infection occurs and the construct is packaged into virus particles, thereby producing packaged virus construct; and

(c) infecting cells with the packaged virus construct.

17. The method of Claim 16 wherein the cell-type-specific promoter is a neural cell-specific promoter.

18. The method of Claim 17 wherein the neural-cell specific promoter is the human neurofilament L promotor.

19. A method of producing tyrosine hydroxylase in a target cell, comprising the steps of:

(a) inserting a nucleotide sequence encoding tyrosine hydroxylase into a defective

Herpes virus vector, the vector having a promoter that is able to express a functional tyrosine hydroxylase gene product in the cell, to form a defective Herpes virus vector construct;

(b) introducing the construct into an appropriate cell line together with a mutant Herpes virus helper virus, whereby a productive infection occurs and the construct is packaged into virus particles, thereby producing packaged virus construct; and

(c) infecting target cells with the packaged virus construct.

20. The method of Claim 19 wherein the mutant helper virus is a deletion virus and the cell line is a complementing cell line.

21. The method of Claim 20 wherein the construct is pHSVth.

22. A method of producing tyrosine hydroxylase in neural cells comprising the steps of:

(a) inserting a nucleotide sequence encoding tyrosine hydroxylase into a defective Herpes virus vector, the vector having a promoter that is able to direct expression of a functional tyrosine hydroxylase product in the cell, to make a defective Herpes virus vector construct;

(b) introducing the construct into an appropriate cell line together with a mutant Herpes helper virus, whereby a productive infection occurs and the construct is packaged into virus particles, thereby producing packaged virus construct; and

(c) infecting neural cells with the packaged virus construct.

23. The method of Claim 22 wherein the defective Herpes virus is HSV-1 the mutant helper Herpes virus is a mutant HSV-1 virus.

24. The method of Claim 23 wherein the construct is pHSVth.

25. A defective Herpes virus vector encoding tyrosine hydroxylase.

26. The vector of Claim 25 wherein the vector is pHSVth.

27. A method of treating or preventing Parkinson's disease in a human comprising administering to the human a packaged defective Herpes virus vector which functionally expresses tyrosine hydroxylase in neural cells in a manner which will deliver the virus particles to cells affected by the disease, whereby infection results in introduction of the vector into the

cells and tyrosine hydroxylase is functionally expressed.

28. A method of altering neurotransmitter metabolism in a neural cell in a mammal, comprising the steps of:

(a) inserting a nucleotide sequence encoding a signal transduction factor gene product or portion thereof into a defective Herpes virus vector, the vector having a promoter that is able to functionally express the gene product in the cell, to make a defective Herpes virus vector construct;

(b) introducing the construct into an appropriate cell line together with a mutant Herpes helper virus, wherein a productive infection occurs and the construct is packaged into virus particles, thereby producing packaged virus construct; and

(c) administering the packaged virus construct to the mammal in a manner which will deliver the virus particles to target cells, whereby infection results in the introduction of the vector into the target cells and the functional expression of the signal transduction factor or portion thereof.

29. The method of Claim 28 wherein the construct is selected from the group consisting of pHSVcyr, pHSVpckΔ, pHSVCaCK, pHSVparv and pDB3.

30. The method of Claim 29 wherein the alteration in neurotransmitter metabolism results in an increase in neurotransmitter release of a neurotransmitter selected from the group consisting of acetylcholine, monoamine neurotransmitters, or excitatory amino acid neurotransmitters.

31. A method of altering neurotransmitter metabolism in a neural cell comprising the steps of:

(a) inserting a nucleotide sequence encoding tyrosine hydroxylase into a defective Herpes virus vector, the vector having a promoter that is able to functionally express the gene product in the cell, to make a defective Herpes virus vector construct;

(b) introducing the construct into an appropriate cell line together with a Herpes virus mutant helper virus, wherein the construct is packaged into virus particles, thereby producing packaged virus construct; and

(c) infecting cells with the packaged virus construct.

32. The method of Claim 31 wherein the construct encoding tyrosine hydroxylase is pHSVth.

33. The method of Claim 31 wherein the alteration in neurotransmitter metabolism results in an increase in monoamine neurotransmitter release.

34. A method of producing a neurotrophic factor in a target cell in a mammal, comprising the steps of:

(a) inserting a nucleotide sequence encoding a neurotrophic factor into a defective Herpes virus vector, the vector having a promoter that is able to express a functional neurotrophic factor gene product in the cell, to form a defective Herpes virus vector construct;

(b) introducing the construct into an appropriate cell line together with a mutant Herpes virus helper virus, whereby a productive infection occurs and the construct is packaged into virus particles, thereby producing packaged virus construct; and

(c) administering the packaged virus construct to the mammal in a manner which will deliver the virus particles to target cells, whereby infection results in the introduction of the vector into the target cells and the functional expression of the neurotrophic factor.

35. The method of Claim 34 wherein the mutant helper virus is a deletion virus, the cell line is a complementing cell line, and the neurotrophic factor is nerve growth factor.

36. The method of claim 35 wherein the construct is pHSVngf.

37. A method of altering neurotransmitter metabolism in a neural cell comprising the steps of:

(a) inserting a nucleotide sequence encoding nerve growth factor into a defective Herpes virus vector, the vector having a promoter that is able to functionally express nerve growth factor in the cell, to make a defective Herpes virus vector construct;

(b) introducing the construct into an appropriate cell line together with a Herpes virus mutant helper virus, wherein the construct is packaged into virus particles, thereby producing packaged virus construct; and

(c) infecting cells with the packaged virus construct.

38. The method of Claim 37 wherein the alteration in neurotransmitter metabolism is an increase in the synthesis of catecholamine or

cholinergic neurotransmitter biosynthetic enzymes.

39. The method of Claim 38 wherein the alteration in neurotransmitter metabolism results in an increase in catecholamine or cholinergic neurotransmitter release.

40. A method of treating or preventing Alzheimer's disease in a human comprising administering to the human a packaged defective Herpes virus vector which functionally expresses nerve growth factor in neural cells in a manner which will deliver the virus particles to target cells, whereby infection results in introduction of the vector into the target cells and nerve growth factor is functionally expressed.

41. A method of treating peripheral nerve injury in a mammal comprising administering to the mammal a packaged defective Herpes virus vector which functionally expresses a neurotrophic factor in neural cells in a manner which will deliver the virus particles to target cells, whereby infection results in introduction of the vector into the target cells and the neurotrophic factor is functionally expressed.

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No	références, formules, pages à photocopier, etc	No	classement
1	Complet (HSV I défectif)	1	C12N15/86D
2	Revendications (Thérapie génique en particulier Parkinson avec Tyrosine hydroxylase)	2	INF A61K48/00
3	(Construction avec NGF) Revendications - Exemples 19-20	3	INF C07K15/00 F24 D10.
4	Revendications - Exemples 1,2,3 14 (construction avec tyrosine hydroxylase)	4.	INF C12N9/02.
5	Revendications - Exemples 17+18 (construction avec proteine kinase C)	5.	INF C12N9/12
6	Revendications Exemples 4,5,6,7,8 9,10,11 (construction avec yeast adenylate cyclase (catalytic domain))	6	INF C12N9/88.
7	Revendications - Exemple 12. (cellules transcomplémentantes du Helper avec IE3)	7	INF C12N5/10.
8	Revendications - Exemple 16. (fusion human GAP-43. pour ciblage ?) (protéine fusion avec ség. signal. 10 aa ~~[illegible]~~)	8	INF C12N15/62A
			M12N 201:00 207:00 M07K 201:00 207:00

Modèle 350

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9107993 SA 53566

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/03/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9006757	28-06-90	CA-A- 2005567	15-06-90
		EP-A- 0449948	09-10-91
EP-A- 0453242	23-10-91	None	
WO-A- 9002551	22-03-90	AU-A- 4327089	02-04-90
		EP-A- 0434750	03-07-91

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 27, 40, 41 and partially 1-26, 28-39, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,9006757 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 June 1990, see the whole document	1-26,28-41
P,Y	EP,A,0453242 (THE GENERAL HOSPITAL CORPORATION) 23 October 1991, see the whole document	1-6,15-17
Y	WO,A,9002551 (BIOSOURCE GENETICS CORPORATION) 22 March 1990, see example 4	1-8,14-20,22,23,25,28,30,31,33

INTERNATIONAL SEARCH REPORT

International Appl No PCT/US 91/07993

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/86 A 61 K 48/00 C 12 N 15/60
C 12 N 15/53 C 12 N 15/54 C 12 N 15/12 C 12 N 15/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	Proc. Natl. Acad. Sci., volume 87, November 1990, Biochemistry (US) A.I. Geller et al.: "An efficient deletion mutant packaging system for defective herpes simplex virus vectors: Potential applications to human gene therapy and neuronal physiology", pages 8950-8954, see the whole article	1-8,14-20,22,23,25,28,30,31,33
X	The New Biologist, volume 2, no. 8, August 1990, Philadelphia, US, E.A. Chiocca et al.: "Transfer and expression of the lacZ gene in rat brain neurons mediated by herpes simplex virus mutants", pages 739-746, see the whole article, especially page 740, column 1, lines 36-40; page 744	1-6,15-17
Y		1-6,15-17

-/-

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26-02-1992	18.03.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Els Vonk

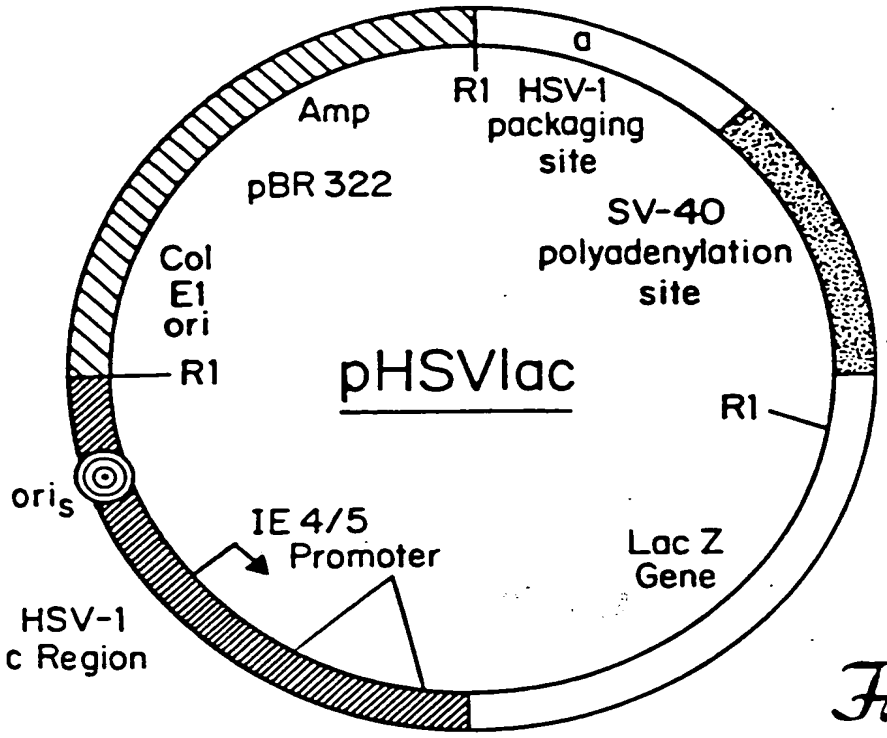
a
R1 HSV-1
packaging
site
Amp
pBR 322
SV-40
polyadenylation
site
Col
E1
ori
R1
pHSVlac
R1
ori$_S$
IE 4/5
Promoter
Lac Z
Gene
HSV-1
c Region

Fig. 1

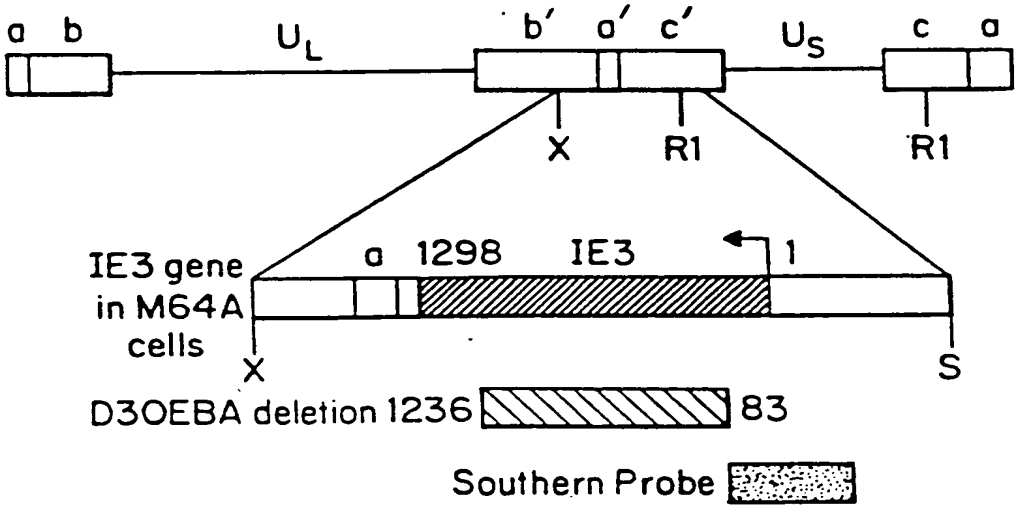
a b
U_L
b′ a′ c′
U_S
c a
X
R1
R1
IE3 gene
in M64A
cells
a 1298 IE3
1
X
S
D3OEBA deletion 1236
83
Southern Probe

Fig. 2

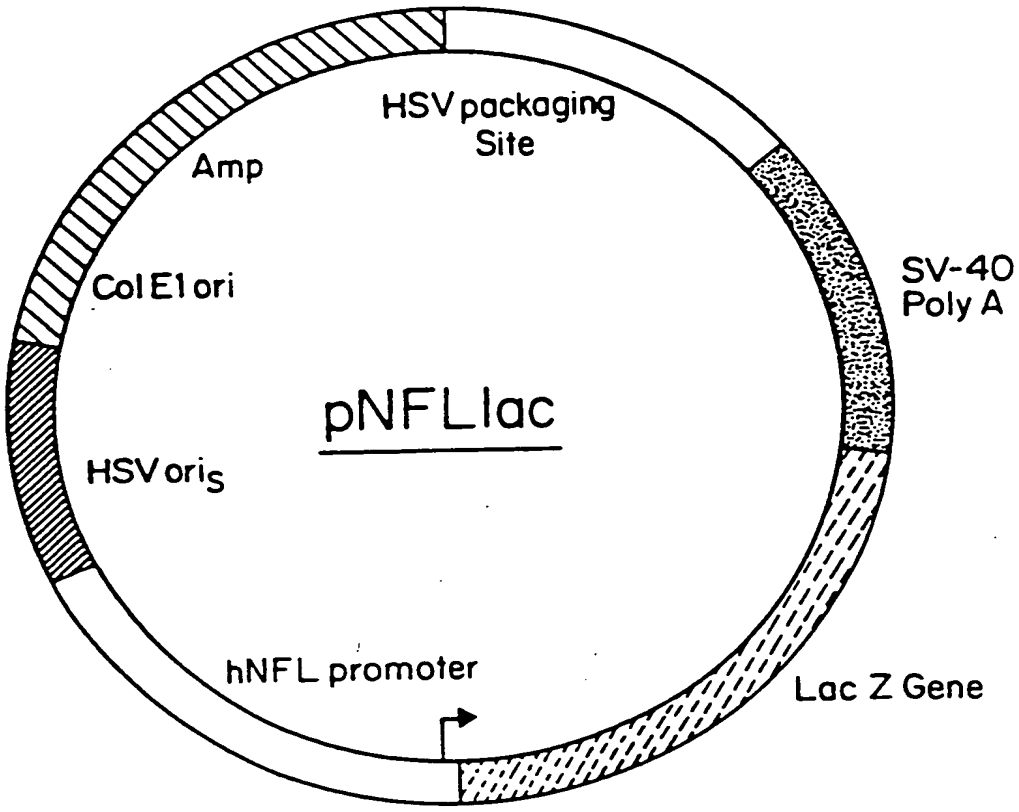
HSV packaging Site
Amp
SV-40 Poly A
ColE1 ori
pNFLlac
HSV oriS
hNFL promoter
Lac Z Gene

Fig. 4

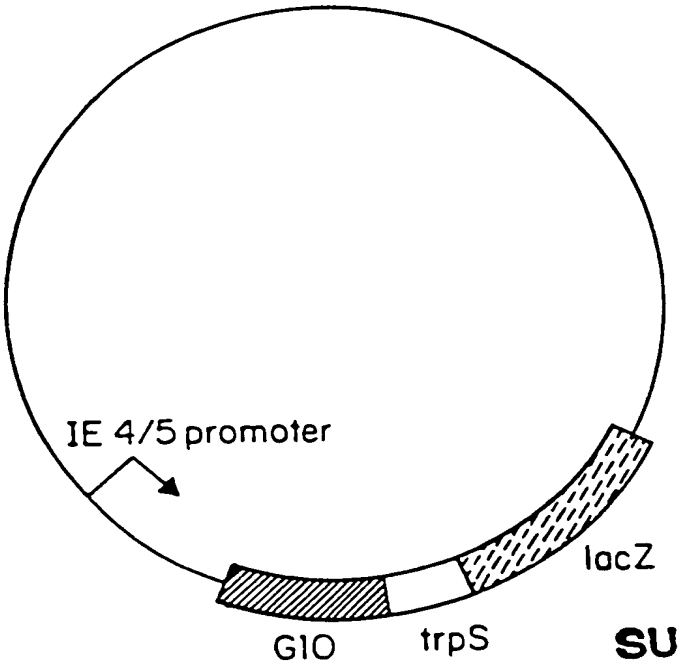
IE 4/5 promoter
lacZ
G10
trpS

Fig. 5

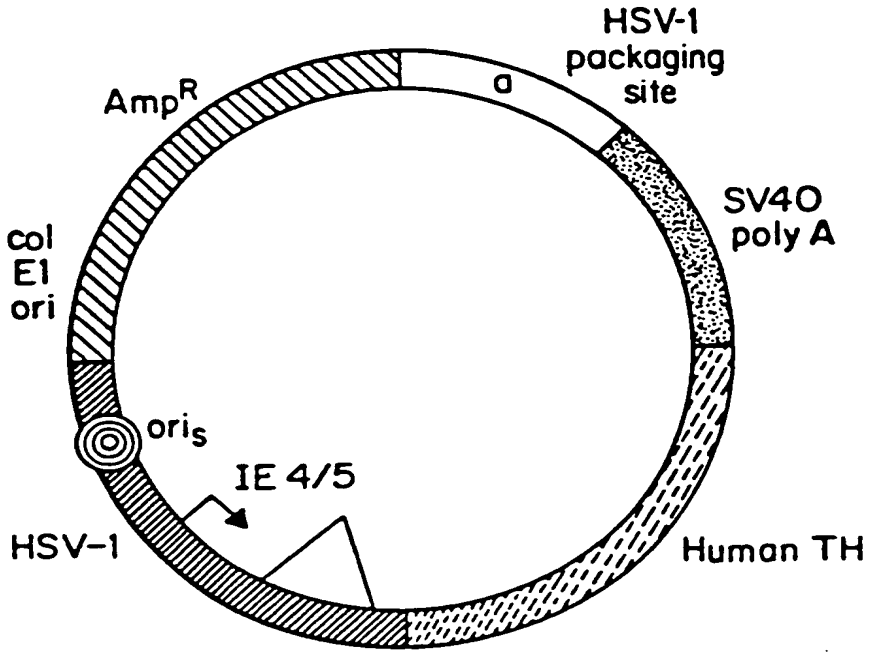
HSV-1
packaging
site
a
Amp^R
SV40
poly A
col
E1
ori
ori$_S$
IE 4/5
HSV-1
Human TH

Fig. 6

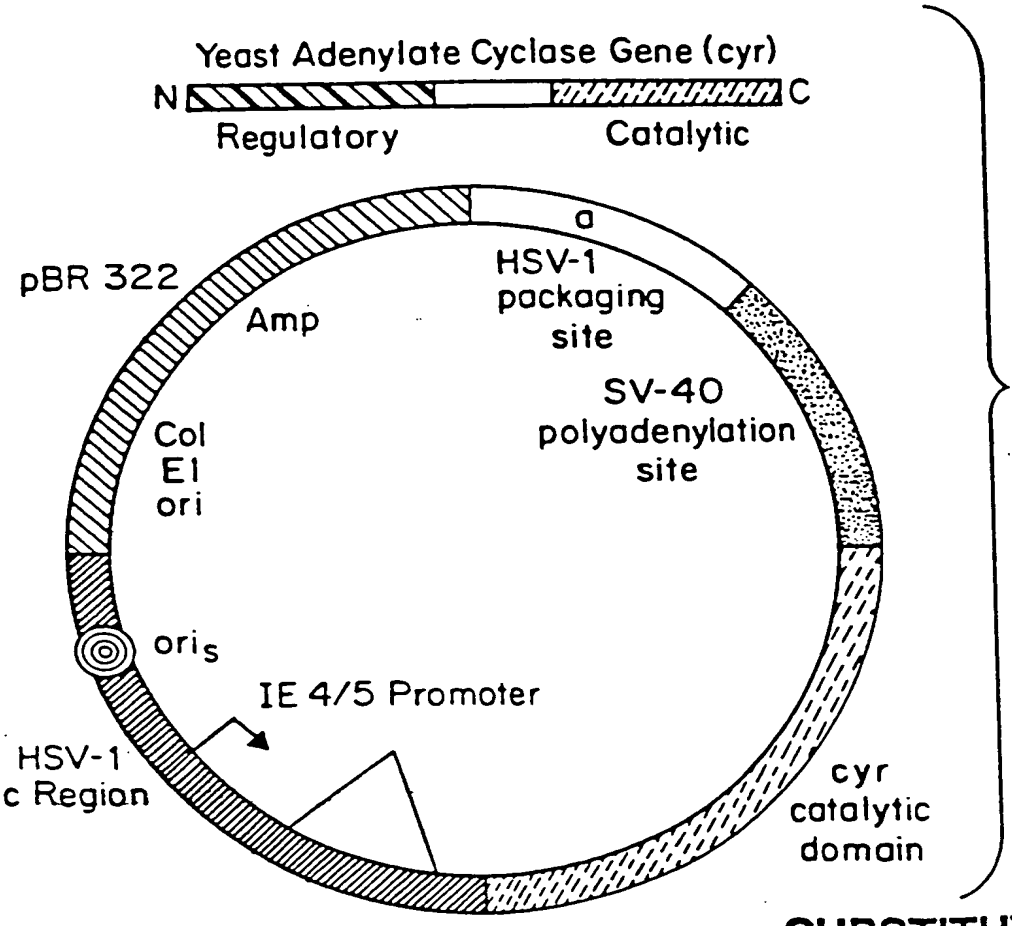
Yeast Adenylate Cyclase Gene (cyr)
N
C
Regulatory
Catalytic
a
pBR 322
HSV-1
packaging
site
Amp
SV-40
polyadenylation
site
Col
E1
ori
ori$_S$
IE 4/5 Promoter
HSV-1
c Region
cyr
catalytic
domain

Fig. 7

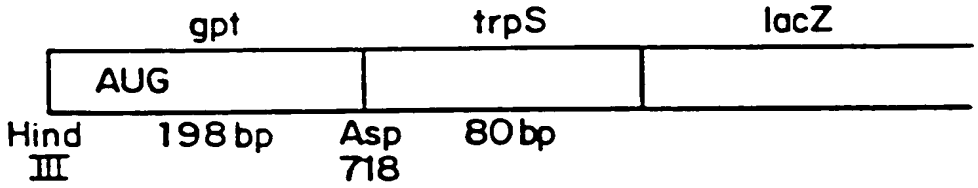
gpt
trpS
lacZ
AUG
Hind III
198 bp
Asp 718
80 bp

Fig. 8

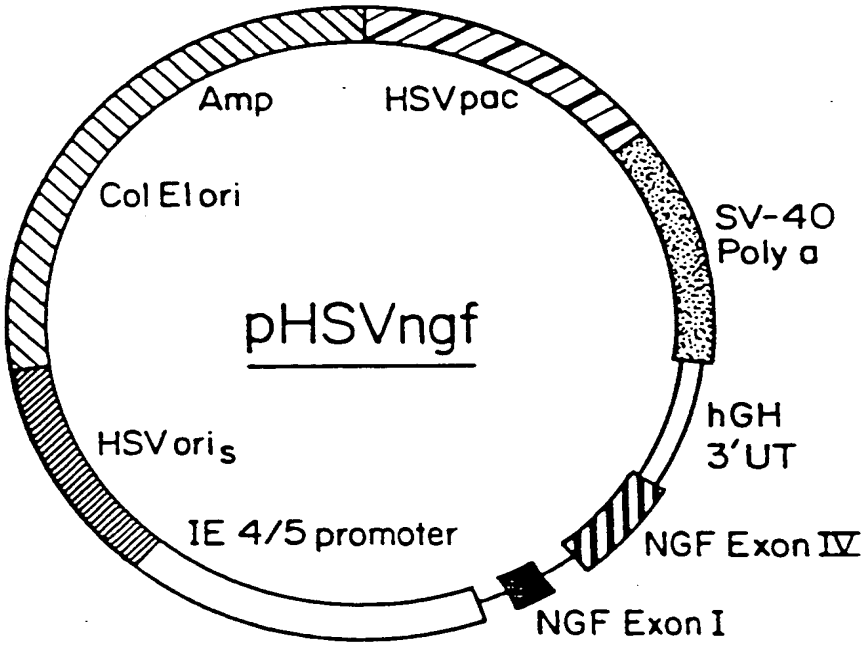
Amp
HSVpac
Col E1 ori
SV-40 Poly a
pHSVngf
hGH 3'UT
HSV ori s
IE 4/5 promoter
NGF Exon IV
NGF Exon I

Fig. 9

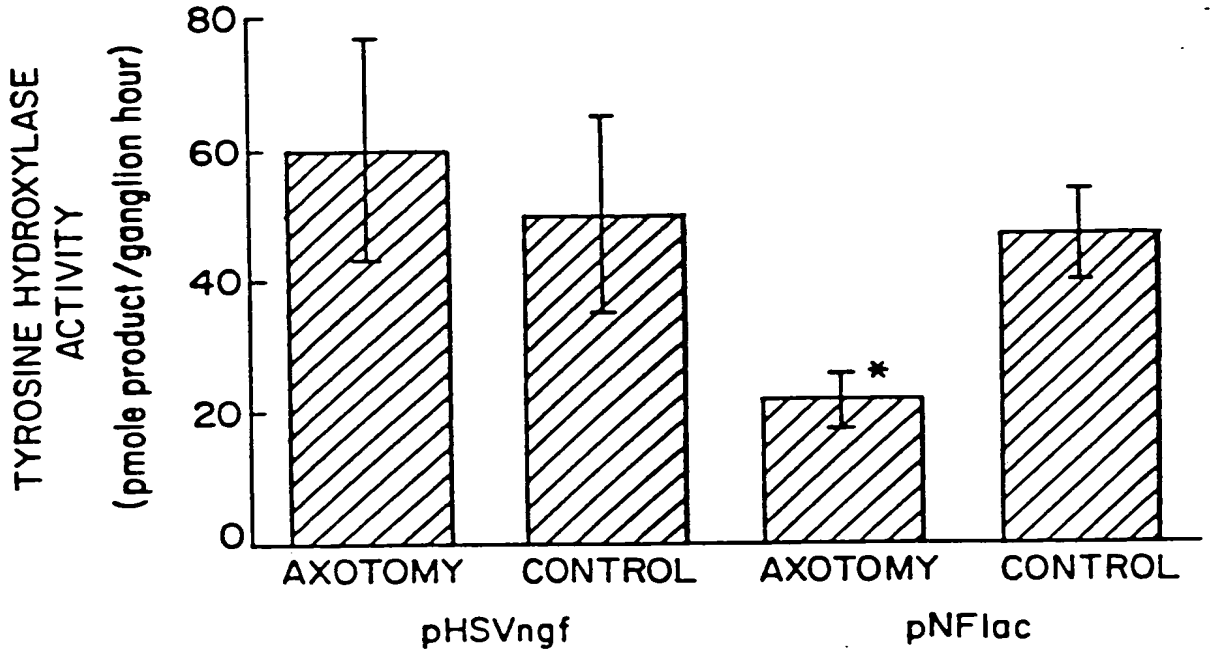
TYROSINE HYDROXYLASE ACTIVITY
(pmole product/ganglion hour)
80
60
40
20
0
*
AXOTOMY
CONTROL
AXOTOMY
CONTROL
pHSVngf
pNFlac

Fig. 10

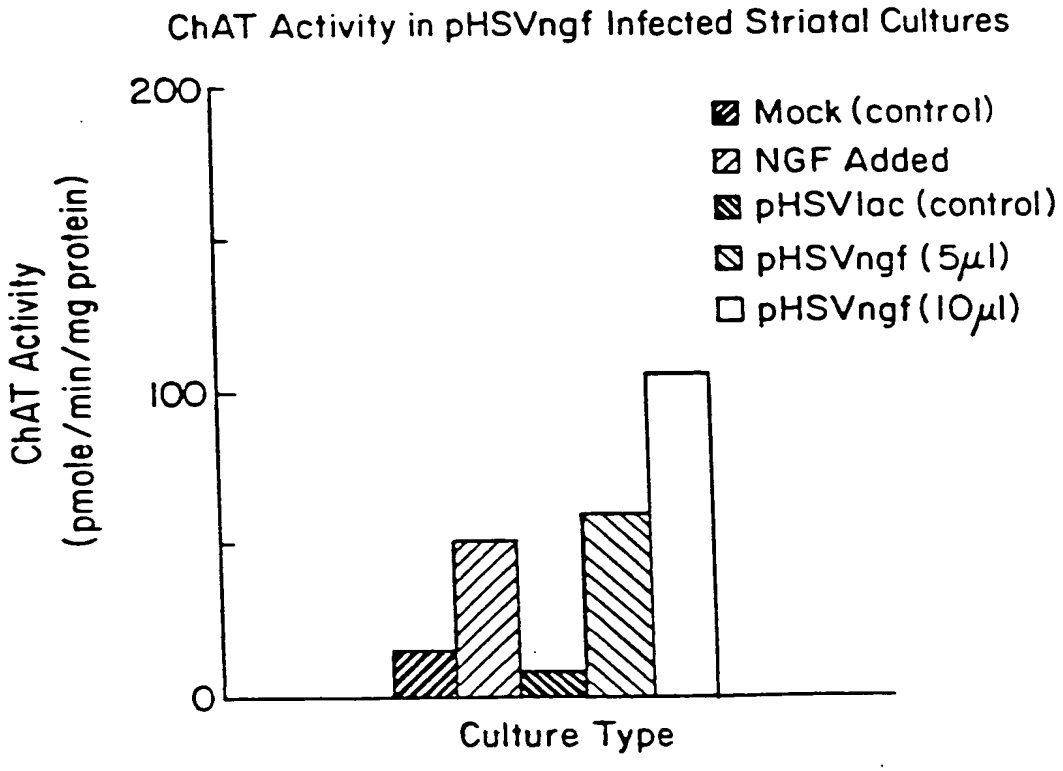
ChAT Activity in pHSVngf Infected Striatal Cultures
ChAT Activity
(pmole/min/mg protein)
200
100
0
Mock (control)
NGF Added
pHSVlac (control)
pHSVngf (5μl)
pHSVngf (10μl)
Culture Type

Fig. 11